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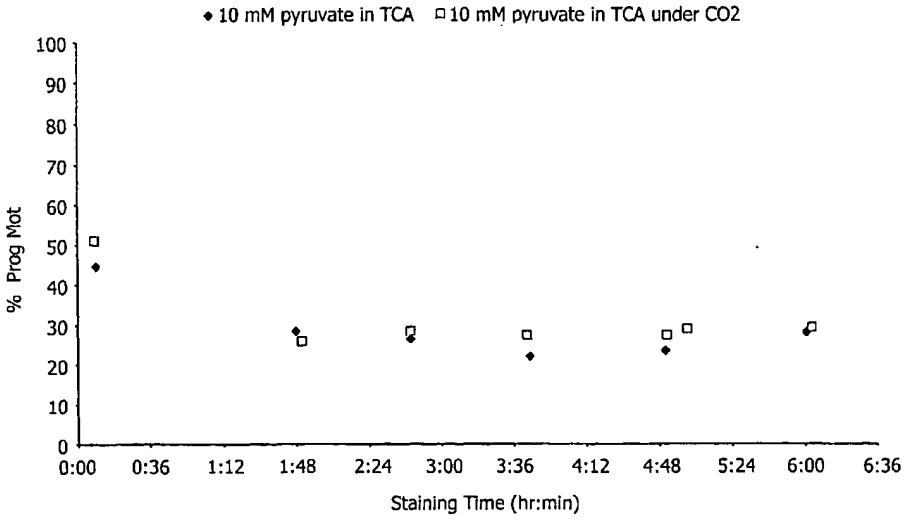
(54) Title: SPERM SUSPENSIONS FOR SORTING INTO X OR Y CHROMOSOME-BEARING ENRICHED POPULATIONS



(57) Abstract: Sperm cell suspensions comprising a motility inhibitor are disclosed. The cells contained in such suspensions tend to have a greater capacity for enduring the various process steps typically associated with the sorting of sperm cells into gender enriched populations, thereby resulting in post-sort compositions with an increased number of viable or motile sperm. Processes for forming such cell suspensions, as well as processes for staining sperm cells, are also disclosed.

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SPERM SUSPENSIONS FOR SORTING INTO X OR Y CHROMOSOME-BEARING ENRICHED POPULATIONS

FIELD OF THE INVENTION

[0001] The present invention generally relates to a process of sorting sperm cells. More specifically, the present invention relates to the preparation of suspensions of sperm cells having reduced motility, and more particularly a temporarily reduced motility, relative to endogenous ejaculated sperm, the suspensions having utility, for example, in a process for sorting sperm cells into an enriched population of X or Y chromosome-bearing sperm cells.

BACKGROUND

[0002] The fertilization of animals by artificial insemination (AI) and embryo transplant following *in vitro* fertilization is an established practice. In the livestock production industry, the ability to influence the reproductive outcome toward offspring having one or more desired characteristics has obvious advantages. By way of example, there would be an economic benefit in the dairy industry to preselect offspring in favor of the female sex to ensure the production of dairy cows. The separation of sperm into enriched populations of X and Y chromosome-bearing cells, known as gender enriched semen or gender enriched sperm, is one method of achieving preselected offspring.

[0003] In order to obtain gender enriched semen, sperm cells must be stained with a dye and subsequently sorted into X and Y chromosome-bearing cells. Each of staining and sorting processes places a stress on the sperm cells that decreases sperm cell viability or motility, particularly progressive motility.

[0004] Salisbury et al. describe a technique for the collection of ejaculated bovine semen directly into a diluent which inhibits cell motility and prevents the absorption of carbohydrates from the surrounding seminal plasma. When the ejaculate is collected into the diluent and the air phase above the liquid is replaced by gassing with 100% CO_2, the cells in the ejaculate became immotile. As long as the cells remained in the diluent and air was excluded, the cells remained immotile for several hours at room temperature and for at least 8 days at 5°C.

Summary of the Invention

[0005] Among the various aspects of the present invention are sperm suspensions having utility, for example, in processes used to sort sperm into enriched populations of X or Y-chromosome bearing sperm.

[0006] Briefly, therefore, the present invention is directed to a sperm cell suspension comprising viable spermatozoa and a composition which down-regulates carbohydrate uptake by the spermatozoa, the concentration of spermatozoa in the suspension being less than about 1×10^6 or at least 1×10^8 spermatozoa per ml.

[0007] The present invention is further directed to a sperm cell suspension comprising viable, immotile sperm, the concentration of spermatozoa in the suspension being less than about 1×10^6 or at least 1×10^8 spermatozoa per ml.

[0008] The present invention is further directed to a sperm cell suspension comprising viable spermatozoa, the spermatozoa having a motility more characteristic of epididymal spermatozoa than endogenous ejaculated spermatozoa of the same species, the concentration of

spermatozoa in the suspension being less than about 1 X 10^6 or at least 1 x 10^8 spermatozoa per ml.

[0009] The present invention is further directed to a sperm cell suspension comprising viable sperm, potassium and optionally sodium, the concentration of spermatozoa in the suspension being at least 1 x 10^8 spermatozoa per ml and the molar ratio of potassium to sodium being greater than 1:1, respectively.

[0010] The present invention is further directed to a sperm cell suspension comprising viable spermatozoa, a composition which down-regulates carbohydrate uptake by the spermatozoa, and a DNA-selective dye.

[0011] The present invention is further directed to a sperm cell suspension comprising viable, immotile sperm and a DNA-selective dye.

[0012] The present invention is further directed to a sperm cell suspension comprising viable spermatozoa and a DNA-selective dye, the spermatozoa having a metabolic rate and motility more characteristic of epididymal spermatozoa than endogenous ejaculated spermatozoa of the same species.

[0013] The present invention is further directed to a sperm cell suspension comprising viable, immotile spermatozoa, the spermatozoa having a DNA-selective dye associated with their DNA.

[0014] The present invention is further directed to a sperm cell suspension comprising viable spermatozoa, the spermatozoa having a metabolic rate and motility more characteristic of epididymal spermatozoa than endogenous ejaculated spermatozoa of the same species, the spermatozoa also having a DNA-selective dye associated with their DNA.

[0015] The present invention is further directed to a process for staining sperm cells, the process comprising forming a staining mixture containing intact viable sperm

cells, a motility inhibiting amount of potassium, and a DNA selective dye.

[0016] The present invention is further directed to a process of forming a sperm cell suspension for use in a flow cytometry process, the process comprising combining a sperm cell source with a composition which inhibits the motility of sperm cells to form a sperm cell suspension, the concentration of sperm cells in the suspension being less than about 1 X 10^6 or at least 1 x 10^8 sperm cells per milliliter.

[0017] The present invention is further directed to a process of forming a sperm cell suspension for use in a flow cytometry process, the process comprising collecting the ejaculate of a mammal in a buffer containing an inhibitory amount of a motility inhibitor to form a sperm cell suspension, the suspension comprising less than about 1 X 10^6 or at least 1 x 10^8 sperm cells per milliliter.

[0018] Other aspects and features of the invention will be in part apparent and in part pointed out hereinafter.

Brief Description of the Drawings

[0019] FIGURE 1 graphically depicts the results of the study carried out in Example 1 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 600μM Hoechst 33342 dye at 28°C in TCA containing 10mM pyruvate or in carbon dioxide-blanketed TCA containing 10mM pyruvate.

[0020] FIGURE 2 graphically depicts the results of the study carried out in Example 1 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 600μM Hoechst 33342 dye at 28°C in TCA containing 10mM pyruvate or a carbonate-based inhibitor at pH 7.3.

[0021] FIGURE 3 graphically depicts the results of the study carried out in Example 1 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 600μM Hoechst 33342 dye at 28°C in TCA containing 10mM pyruvate or a carbonate-based inhibitor at pH 6.2.

[0022] FIGURE 4 graphically depicts the results of the study carried out in Example 2 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 1000μM Hoechst 33342 dye at 28°C in TCA containing 10mM pyruvate and then diluted 1 to 3 with either TCA containing 10mM pyruvate or a carbonate-based inhibitor at pH 6.2.

[0023] FIGURE 5 graphically depicts the results of the study carried out in Example 2 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 1000μM Hoechst 33342 dye at 28°C in (1) TCA containing 10mM pyruvate and diluted 1 to 3 with the same or (2) a carbonate-based buffer at pH 7.3 and diluted 1 to 3 with carbonate-based inhibitor at pH 6.2.

[0024] FIGURE 6 graphically depicts the results of the study carried out in Example 2 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 1000μM Hoechst 33342 dye at 28°C in TCA containing 10mM pyruvate or a carbonate-based inhibitor at pH 6.2.

[0025] FIGURE 7 graphically depicts the results of the study carried out in Example 3 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 300μM Hoechst 33342 dye at 41°C in TCA containing 10mM pyruvate and then diluted 1 to 3 with either TCA containing 10mM pyruvate or a carbonate-based inhibitor at pH 6.2.

[0026] FIGURE 8 graphically depicts the results of the study carried out in Example 3 wherein percent progressive motility of sperm cells is measured for sperm cells stained

with 300μM Hoechst 33342 dye at 41°C in (1) TCA containing 10mM pyruvate and diluted 1 to 3 with the same or (2) a carbonate-based buffer at pH 7.3 and diluted 1 to 3 with carbonate-based inhibitor at pH 6.2.

[0027] FIGURE 9 graphically depicts the results of the study carried out in Example 3 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 300μM Hoechst 33342 dye at 41°C in TCA containing 10mM pyruvate or a carbonate-based inhibitor at pH 6.2.

[0028] FIGURE 10 graphically depicts the results of the study carried out in Example 4 wherein percent progressive motility of sperm is measured for sperm stained with 400μM Hoechst 33342 dye at 41°C in either a TCA buffer or a TCA buffer containing 10mM pyruvate.

[0029] FIGURE 11 graphically depicts the results of the study carried out in Example 5 wherein percent progressive motility of sperm is measured for sperm stained with 400μM Hoechst 33342 dye at 41°C in either a TCA buffer or a TCA buffer containing 10μM vitamin K.

[0030] FIGURE 12 graphically depicts the results of the study carried out in Example 6 wherein percent progressive motility of sperm is measured for sperm stained with 400μM Hoechst 33342 dye at 41°C in either a TCA buffer or a TCA buffer containing 100μM vitamin K.

[0031] FIGURE 13 graphically depicts the results of the study carried out in Example 7 wherein percent progressive motility of sperm is measured for sperm stained with 400μM Hoechst 33342 dye at 41°C in either a TCA buffer or a TCA buffer containing 1mM lipoic acid.

[0032] FIGURE 14 graphically depicts the results of the study carried out in Example 8 wherein percent progressive motility of sperm is measured for sperm stained

with 600μM Hoechst 33342 dye at 28°C in either a TCA buffer or a TCA buffer containing 10mM pyruvate.

[0033] FIGURE 15 graphically depicts the results of the study carried out in Example 9 wherein percent progressive motility of sperm is measured for sperm stained with 600μM Hoechst 33342 dye at 28°C in either a TCA buffer or a TCA buffer containing 100μM vitamin K.

[0034] FIGURE 16 graphically depicts the results of the study carried out in Example 10 wherein percent progressive motility of sperm is measured for sperm stained with 600μM Hoechst 33342 dye at 28°C in either a TCA buffer or a TCA buffer containing 1mM lipoic acid.

[0035] FIGURE 17 graphically depicts the results of the study carried out in Example 11 wherein percent progressive motility of sperm is measured for sperm stained with 600μM Hoechst 33342 dye at 28°C in a TCA buffer, a TCA buffer containing 2.5mM pyruvate, a TCA buffer containing 10mM pyruvate, a TCA buffer containing 25mM pyruvate, and a TCA buffer containing 50mM pyruvate.

[0036] FIGURE 18 graphically depicts the results of the study carried out in Example 12 wherein percent progressive motility of sperm is measured for sperm stained with 20μM SYBR-14 dye at 28°C in either a TCA buffer or a TCA buffer containing 10mM pyruvate.

[0037] FIGURE 19 graphically depicts the results of the study carried out in Example 13 wherein percent progressive motility of sperm is measured for sperm stained with 100μM BBC dye at 28°C in either a TCA buffer or a TCA buffer containing 10mM pyruvate.

[0038] FIGURE 20 graphically depicts the results of the study carried out in Example 14 wherein percent progressive motility of sperm is measured for sperm stained

with 200μM BBC dye at 28°C in either a TCA buffer or a TCA buffer containing 10mM pyruvate.

Detailed Description of the Invention

[0039] Surprisingly, it has been determined that spermatozoa having reduced motility relative to endogenous ejaculated spermatozoa (of the same species) tend to have a greater capacity for enduring the various process steps typically associated with the sorting of sperm cells into an enriched population of X or Y chromosome-bearing spermatozoa. In a preferred embodiment, therefore, gender enriched populations of spermatozoa may be prepared for artificial insemination which have an increased number of viable cells or an increased number of motile sperm, particularly progressively motile sperm, in a post-stain or post-sort composition.

[0040] In accordance with the process of the present invention, a suspension, sometimes referred to as a dispersion, is formed containing spermatozoa and one or more compositions which inhibit the motility of the spermatozoa; such a state of inhibited motility sometimes being referred to as immotility or sperm quiescence. In general, the suspensions will contain spermatozoa in a density of about 1 X 10^3 sperm/ml to about 5 X 10^{10} sperm/ml of suspension. For example, in one embodiment the suspensions may contain spermatozoa in a "relatively low" density, *i.e.*, in a density of less than about 1 X 10^7 sperm/ml, preferably less than about 1 X 10^6 sperm/ml, more preferably about 1 X 10^3 to about 5 X 10^6 sperm/ml, still more preferably about 1 X 10^3 to about 1 X 10^6 sperm/ml, even more preferably about 1 X 10^4 to about 1 X 10^5 sperm/ml, and most preferably about 1 X 10^5 sperm/ml of

suspension. In an alternative embodiment, the suspensions may contain spermatozoa in an "intermediate" density, *i.e.*, in a density of about 1 X 10^7 to about 1 X 10^8 sperm/ml of suspension. In yet another alternative embodiment, the suspensions may contain spermatozoa in a "relatively high" density, *i.e.*, in a density of at least about 1 x 10^8 sperm/ml, preferably about 1 X 10^8 to about 5 X 10^{10} sperm/ml, more preferably about 1.5 X 10^8 to about 2 X 10^{10} sperm/ml, even more preferably about 1.5 X 10^8 to about 2 X 10^8 sperm/ml, and still more preferably about 1.5 X 10^8 sperm/ml of suspension. Thus, for example, in one embodiment the suspension may contain at least about 1.25 x 10^8, at least about 1.5 x 10^8, at least about 1.75 x 10^8, at least about 2 x 10^8, at least about 2.25 x 10^8, at least about 2.5 x 10^8, at least about 2.75 x 10^8, or even at least about 3 x 10^8 sperm/ml of suspension. In an alternative embodiment, the suspension may contain less than about 9 X 10^5, less than about 7 X 10^5, less than about 5 X 10^5, less than about 2 X 10^5, less than about 1 X 10^5, less than about 1 X 10^4, or even less than about 1 X 10^3 sperm/ml of suspension.

[0041] The density of spermatozoa in the sperm suspensions depends upon several considerations, including the method by which the sperm cells may be subsequently enriched or sorted. For example, the sperm cells may be sorted using flow cytometry as described in greater detail below. In such an instance, the buffered sperm suspension may typically be of an "intermediate" or "relatively high" density of spermatozoa. Other sorting or enrichment techniques may benefit from a lesser density of spermatozoa, such as a "relatively low" density of spermatozoa, labeled with a marker, such as for example the dyes and labels described herein.

[0042] In a preferred embodiment, spermatozoa in suspensions of the present invention behave, in certain respects, in a manner characteristic of epididymal spermatozoa; for example, the spermatozoa may be immotile and/or they may have a lesser rate of endogenous respiration and a greater rate of aerobic glycolysis as compared to washed or freshly ejaculated spermatozoa. Advantageously, the inhibited spermatozoa have the ability, upon separation from the inhibitor(s), to behave in a manner characteristic of ejaculated spermatozoa (and not characteristic of epididymal spermatozoa) with respect to motility and, in one embodiment, with respect to motility and respiration.

[0043] In one embodiment, for example, the motility inhibitor reduces path velocity, progressive velocity, or both, as measured by HTM-IVOS sperm analysis (Hamilton-Thorne HTM-IVOS computer assisted sperm analysis system Hamilton-Thorne Research, Beverly MA) of at least about 50% of the sperm cells in the dispersion relative to the path velocity, progressive velocity, or both of sperm cells in a fresh ejaculate of the same species. Preferably, the motility inhibitor reduces path velocity, progressive velocity, or both, as measured by HTM-IVOS sperm analysis, of at least about 60% of the sperm cells in the dispersion relative to the path velocity, progressive velocity, or both of sperm cells in a fresh ejaculate of the same species. More preferably, the motility inhibitor reduces path velocity, progressive velocity, or both, as measured by HTM-IVOS sperm analysis, of at least about 70% of the sperm cells in the dispersion relative to the path velocity, progressive velocity, or both of sperm cells in a fresh ejaculate of the same species. Still more preferably, the motility inhibitor reduces path velocity,

progressive velocity, or both, as measured by HTM-IVOS sperm analysis, of at least about 80% of the sperm cells in the dispersion relative to the path velocity, progressive velocity, or both of sperm cells in a fresh ejaculate of the same species. Even more preferably, the motility inhibitor reduces path velocity, progressive velocity, or both, as measured by HTM-IVOS sperm analysis, of at least about 90% of the sperm cells in the dispersion relative to the path velocity, progressive velocity, or both of sperm cells in a fresh ejaculate of the same species. Even more preferably, the motility inhibitor reduces path velocity, progressive velocity, or both, as measured by HTM-IVOS sperm analysis, of at least about 95% of the sperm cells in the dispersion relative to the path velocity, progressive velocity, or both of sperm cells in a fresh ejaculate of the same species. Most preferably, the motility inhibitor reduces path velocity, progressive velocity, or both, as measured by an HTM-IVOS sperm analysis, of at least about 99% of the sperm cells in the dispersion relative to the path velocity, progressive velocity, or both of sperm cells in a fresh ejaculate of the same species.

[0044] In addition to or in lieu of an inhibitory buffer, the temperature of the sperm cells or the immediate environment surrounding the sperm cells (i.e., a sperm dispersion) may solely be reduced to affect the motility of the cells. Such a reduction in temperature will generally increase immotility. Moreover, for example, the reduction of temperature of the sperm cells or the sperm dispersion may permit a reduction in the concentration of inhibitor used to induce immotility. Accordingly, the sperm dispersion may be at a temperature not in excess of 5°C; preferably between about 0°C and about 5°C; more preferably between about 3°C and about 5°C; and most preferably about

5°C. Alternatively, the sperm dispersion may be at a temperature within the range of about 4°C to about 50°C; preferably from about 7°C to about 43°C; more preferably from about 10°C to about 39°C; still more preferably from about 15°C to about 30°C; even more preferably from about 17°C to about 25°C; and most preferably at about 18°C. Preferably, however, the sperm cells are not exposed to temperatures that substantially detrimentally affect the viability of the cells.

[0045] The inhibitor may be any of a range of compositions having a depressive effect upon sperm motility. Such compositions include, for example, sodium/potassium ATPase inhibitors, such as, ouabain; compositions comprising potassium ions; and compositions comprising potassium and sodium ions. For example, relatively high concentrations of potassium ions in the suspension tend to depress sperm motility. In general, therefore, it is preferred that the suspension contain a source of potassium ions and that the potassium concentration in the suspension be at least about 0.05 moles/L. More preferably, the potassium concentration is at least about 0.05 moles/L to about 0.5 moles/L. Still more preferably, the potassium concentration is at least about 0.1 moles/L to about 0.3 moles/L. Most preferably, the potassium concentration is at about 0.173 moles/L. Such suspensions will typically, but not necessarily, also contain a source of sodium ions. When sodium is present, the molar ratio of potassium to sodium is generally equal to or greater than 1:1, respectively. Preferably, the molar ratio of potassium to sodium is at least about 1.25:1. Still more preferably, the molar ratio of potassium to sodium is at least about 1.5:1. Still more preferably, the molar ratio of potassium to sodium is at

least about 1.75:1. Still more preferably, the molar ratio of potassium to sodium is at least about 1.78:1. In one particular embodiment, the molar ration of potassium to sodium is at least about 2:1. In yet another embodiment, the molar ratio of potassium to sodium is at least about 3:1. In still another embodiment, the molar ratio of potassium to sodium is at least about 4:1. In still another embodiment, the molar ratio of potassium to sodium is at least about 5:1. In still another embodiment, the molar ratio of potassium to sodium is at least about 6:1. In still another embodiment, the molar ratio of potassium to sodium is at least about 7:1. In still another embodiment, the molar ratio of potassium to sodium is at least about 8:1.

[0046] The sperm suspension may additionally comprise an ion or source of carbon dioxide capable of down-regulating uptake of carbohydrate. In this embodiment, the source of carbon dioxide may be, for example, one or more carbonates. In one presently preferred embodiment, the sperm suspension comprises $NaHCO_3$ and $KHCO_3$, thereby providing a source potassium and sodium ions as well as a partial pressure of carbon dioxide. For example, in one presently preferred embodiment, the suspension comprises $NaHCO_3$ and $KHCO_3$ in an aqueous solution, preferably $NaHCO_3$, $KHCO_3$, and $C_6H_8O_7 \cdot H_2O$ in water; In general, the $KHCO_3$ concentration in the dispersion may be at least about 0.05 moles/L. More preferably, the $KHCO_3$ concentration is at least about 0.05 moles/L to about 0.5 moles/L. Still more preferably, the $KHCO_3$ concentration is at least about 0.1 moles/L to about 0.3 moles/L. In one particularly preferred embodiment, the suspension is formed using an inhibitory buffer comprising 0.097 moles/L of $NaHCO_3$, 0.173 moles/L of $KHCO_3$, 0.090 moles/L $C_6H_8O_7 \cdot H_2O$ in water as

disclosed in Salisbury & Graves, *J. Reprod. Fertil.*, 6:351-359 (1963). The sperm cells will generally remain quiescent as long as they are exposed to the motility inhibitor(s).

[0047] When $C_6H_8O_7 \cdot H_2O$ is present in the dispersion, the molar ratio of $KHCO_3$ to $NaHCO_3$ may be as described above. The molar ratio of $KHCO_3$ to $C_6H_8O_7 \cdot H_2O$ may generally be equal to or greater than 1:1, respectively, but will generally not exceed a molar ratio of 8:1. Preferably, the molar ratio of $KHCO_3$ to $C_6H_8O_7 \cdot H_2O$ is from at least about 1.25:1. Still more preferably, the molar ratio of $KHCO_3$ to $C_6H_8O_7 \cdot H_2O$ is at least about 1.5:1. Still more preferably, the molar ratio of $KHCO_3$ to $C_6H_8O_7 \cdot H_2O$ is at least about 1.75:1. In one particular embodiment, the molar ratio of $KHCO_3$ to $C_6H_8O_7 \cdot H_2O$ is at least about 1.78:1. In another particular embodiment, the molar ratio of $KHCO_3$ to $C_6H_8O_7 \cdot H_2O$ is at least about 2:1. In yet another embodiment, the molar ratio of $KHCO_3$ to $C_6H_8O_7 \cdot H_2O$ is at least about 3:1. In still another embodiment, the molar ratio of $KHCO_3$ to $C_6H_8O_7 \cdot H_2O$ is at least about 4:1. In still another embodiment, the molar ratio of $KHCO_3$ to $C_6H_8O_7 \cdot H_2O$ is at least about 5:1. In still another embodiment, the molar ratio of $KHCO_3$ to $C_6H_8O_7 \cdot H_2O$ is at least about 6:1. In still another embodiment, the molar ratio of $KHCO_3$ to $C_6H_8O_7 \cdot H_2O$ is at least about 7:1. In still another embodiment, the molar ratio of $KHCO_3$ to $C_6H_8O_7 \cdot H_2O$ is at least about 8:1. In one particularly preferred embodiment, the dispersion is formed using an inhibitory buffer comprising 0.097 moles/L of $NaHCO_3$, 0.173 moles/L of $KHCO_3$, 0.090 moles/L $C_6H_8O_7 \cdot H_2O$ in water as disclosed in Salisbury & Graves, *J. Reprod. Fertil.*, 6:351-359 (1963). The sperm cells will generally remain quiescent as long as they are exposed to the motility inhibitor(s).

[0048] Experimental evidence to date further suggests that the overall health and other vital characteristics of sperm cells may be improved if the cell suspension is maintained under an atmosphere having an enhanced partial pressure of carbon dioxide relative to air. In a preferred embodiment, the atmosphere over the suspension has a partial pressure of carbon dioxide of at least 0.9; more preferably, at least about 0.95.

[0049] Quiescent cells may be returned to an active state by separating the cells from the motility inhibitor and exposing them to air. In addition, the initiation of an active state may be further induced by the dilution of the cells in a physiological saline (Salisbury et al., 1963) or a buffer such as TCA buffer or PBS. Typically, at least about 20%, preferably at least about 50%, more preferably at least about 60%, still more preferably at least about 70%, even more preferably at least about 80%, even more preferably at least about 90%, still more preferably at least about 95%, and most preferably at least about 99% of the cells returned to an active state (*i.e.*, reactivated cells) will have a path velocity, progressive velocity, or both, as measured by HTM-IVOS sperm analysis, that is at least about 50%, preferably at least about 60%, more preferably at least about 70%, still more preferably at least about 80%, even more preferably at least about 90%, even more preferably at least about 95%, and most preferably at least about 99% of the path velocity, progressive velocity, or both of the sperm cells prior to being combined with the motility inhibitor (*i.e.*, of sperm cells of a fresh ejaculate).

[0050] In general, the cell sorting process comprises a series of discrete steps, *i.e.*, collection of a cell sample, staining of the cells, sorting of the cells,

collection of the sorted cells, and optionally, cryoextension of the sorted cells. Advantageously, the motility inhibitor may be included in sperm suspensions formed or employed in one or more of these steps.

Collection of the cell sample

[0051] Intact viable bovine, porcine, equine, or other mammalian sperm cells, may be collected and contacted with the motility inhibitor. Various methods of collection of viable sperm are known and include, for example, the gloved-hand method, use of an artificial vagina, and electro-ejaculation. As an example, a bovine semen sample, typically containing about 0.5 to about 10 billion sperm cells per milliliter, may be collected directly from the source mammal into a vessel containing a motility inhibitor to form a sperm suspension. Alternatively, the semen sample may be collected into an empty vessel and then subsequently contacted with the motility inhibitor within several minutes to hours after collection to form the sperm suspension.

[0052] In addition to a buffer, the sperm suspension may also contain a range of additives to enhance sperm viability. Exemplary additives include protein sources, antibiotics, and compositions which regulate oxidation/reduction reactions intracellularly and/or extracellularly.

[0053] Exemplary protein sources include egg yolk, egg yolk extract, milk (including heat homogenized and skim), milk extract, soy protein, soy protein extract, serum albumin, bovine serum albumin, human serum substitute supplement, and combinations thereof. Albumin, and more particularly bovine serum albumin (BSA), is a preferred protein source. For example, if included, BSA may be

present in the sperm suspension in an amount of less than about 5.0% (w/v), preferably less than about 2% (w/v), more preferably less than about 1% (w/v), and most preferably in an amount of about 0.1% (w/v).

[0054] The use of a protein source, such BSA, alone may initiate the process of capacitation in a percentage of the sperm cells in the suspension. It is preferred that this process take place in the female reproductive tract. Therefore, in order to inhibit the initiation of capacitation during dilution, as well as during the subsequent staining and sorting, an alternative protein source or a protein substitute may be included in the sperm suspension. The alternative protein source or protein substitute possess the advantageous effects of a typical protein source, such as BSA, in addition to the ability to inhibit the initiation of capacitation in a larger percentage of the cells in the sperm suspension. Examples of a alternative protein sources includes human serum substitute supplement (SSS) (Irvine Scientific, Santa Ana, CA) and cholesterol enhanced BSA, while an example of a protein substitute includes a polyvinyl alcohol, such as for example, a low to medium viscosity polyvinyl alcohol generally of a molecular weight of about 30,000 to about 60,000. Generally, if included, these compositions will be present in the same amounts as disclosed above with respect to BSA, with the total albumin content of the buffer or buffered solution generally not exceeding about 5.0% (w/v).

[0055] Exemplary compositions which regulates oxidation/reduction reactions intracellularly and/or extracellularly include for example pyruvate, vitamin K, lipoic acid, glutathione, flavins, quinones, superoxide dismutase (SOD), and SOD mimics. If included in the sperm suspension, such a composition may be present in a

concentration sufficient to effect the protective effect without detrimentally affecting sperm health. Exemplary concentration ranges include from about 10μM to about 20mM depending upon such factors as the particular composition being used or the concentration of sperm in the suspension. For example, pyruvate may be present in the sperm suspension in a concentration from about 1mM to about 20mM, preferably from about 5mM to about 15mM, and more preferably about 10mM. Vitamin K may be present in the sperm suspension in a concentration from about 1μM to about 100μM, preferably from about 10μM to about 100μM, and more preferably about 100μM. Lipoic acid may be present in the sperm suspension in a concentration from about 0.1mM to about 1mM, preferably from about 0.5mM to about 1mM, and more preferably about 1mM.

[0056] An antibiotic may be included in the sperm suspension in order to inhibit bacterial growth. Exemplary antibiotics include, for example, tylosin, gentamicin, lincomycin, spectinomycin, Linco-Spectin® (lincomycin hydrochloride-spectinomycin), penicillin, streptomycin, ticarcillin, or any combination thereof. If included, the antibiotics may be present in a concentration of about 50μg to about 800μg per ml of semen, regardless of whether the semen is neat, buffered, or contains additional substances, such as for example, any of the additives mentioned herein. The Certified Semen Services (CSS) and National Association of Animal Breeders (NAAB) have promulgated guidelines regarding the use of antibiotics with respect to sperm collection and use.

[0057] A growth factor may be added to the sperm dispersion in order to help maintain the viability of the sperm cells. Exemplary growth factors include, for

example, transforming growth factors ("TGF), such as, for example, TGFβ-1 and TGFβ-2, and insulin-like growth factors ("IGF"), such as for example, IGF-1. Generally, TGF may be present in the sperm dispersion in the form of TGFβ-1 in a concentration of about 0.1ng/L to about 10μg/L or as TGFβ-2 in a concentration of about 0.1ng/L to about 200ng/L, and IGF may be present in the sperm dispersion in the form of IGF-1 in a concentration of about 0.1ng/L to about 50μg/L. The use of such growth factors is well known in the art and is disclosed, for example, in U.S. Patent Application Publication No. 2003/0157473, the content of which is hereby incorporated herein by reference.

[0058] Once collected, the cells may be stored in a quiescent state for several hours at room temperature, for several weeks at a reduced temperature, such as for example at 5°C, or stored for several months in a cryoextender as discussed below. Preferably, the atmosphere above the cells has a high partial pressure of CO_2 as discussed above. Alternatively, the collected cells may be used within several hours, such as for example in a fertilization process, a staining process, or a sorting process.

Staining of the cells

[0059] A motility inhibitor may be used to render cells immotile during staining of the cells. A process of staining sperm cells typically comprises the formation of a staining mixture, sometimes referred to as a labeling mixture, containing intact viable sperm cells, a motility inhibitor, and a dye, sometimes referred to as a label. In this aspect of the invention, the motility inhibitor may be contacted with the sperm cells to form a sperm suspension, and then the suspension contacted with a DNA selective dye. In this embodiment, the sperm source may be neat semen, or

alternatively, a sperm-containing semen derivative obtained by centrifugation or the use of other means to separate semen into fractions.

[0060] In an alternative embodiment, the dye may be combined with a motility inhibitor, thereby forming a dye solution. Thus, for example, dye in the form of a neat solid, including a free-flowing powder, or a liquid composition may be combined with the inhibitor to form a dye solution, which may then be combined with neat semen, a sperm suspension, or a sperm-containing semen derivative.

[0061] In any event, the sperm cells will generally remain quiescent as long as they are maintained in the inhibitor. (Salisbury et al., 1963) Preferably, however, the staining mixture is maintained under an atmosphere having an enriched partial pressure of carbon dioxide relative to air; for example, providing an atmosphere over the staining mixture which is 99%+ CO_2 is generally preferred.

[0062] The pH of the staining mixture may be maintained at any of a range of pH's; typically this will be in the range of about 5.0 to about 9.0. For example, the staining mixture may be maintained at a "slightly acid" pH, *i.e.*, from about 5.0 to about 7.0 . In this embodiment, the pH is preferably from about 6.0 to about 7.0, more preferably from about 6.0 to about 6.5, and most preferably at about 6.2. Alternatively, the staining mixture may be maintained at a "slightly basic" pH, *i.e.*, from about 7.0 to about 9.0. In this embodiment, the pH is preferably from about 7.0 to about 8.0, more preferably from about 7.0 to about 7.5, and most preferably at about 7.3.

[0063] The staining mixture may be formed by using one or more UV or visible light excitable , DNA selective dyes as previously described in U.S. Patent No. 5,135,759 and

WO 02/41906. Exemplary UV light excitable, selective dyes include Hoechst 33342 and Hoechst 33258 , each of which is commercially available from Sigma-Aldrich (St. Louis, MO). Exemplary visible light excitable dyes include SYBR-14, commercially available from Molecular Probes, Inc. (Eugene, OR) and bisbenzimide-BODIPY® conjugate 6-{[3-((2Z)-2-{[1-(difluoroboryl)-3,5-dimethyl-1*H*-pyrrol-2-yl]methylene}-2*H*-pyrrol-5-yl)propanoyl]amino}-*N*-[3-(methyl{3-[({4-[6-(4-methylpiperazin-1-yl)-1*H*,3'*H*-2,5'-bibenzimidazol-2'-yl]phenoxy}acetyl)amino]propyl}amino)propyl]hexanamide ("BBC") described in WO 02/41906. Each of these dyes may be used alone or in combination; alternatively, other cell permeant UV and visible light excitable dyes may be used, alone or in combination with the aforementioned dyes, provided the dye does not detrimentally affect the viability of the sperm cells to an unacceptable degree when used in concentrations which enable sorting as described elsewhere.

[0064] Alternatively, the staining mixture may be formed using fluorescent polyamides, and more specifically polyamides with a fluorescent label or reporter conjugated thereto. Such labels will fluoresce when bound to nucleic acids. Examples of polyamides with a fluorescent label or reporter attached thereto include, for example, those disclosed in Best et al., *Proc. Natl. Acad. Sci. USA*, 100(21): 12063-12068 (2003); Gygi, et al., *Nucleic Acids Res.*, 30(13): 2790-2799 (2002); U.S. Patent No. 5,998,140; U.S. Patent No. 6,143,901; and U.S. Patent No. 6,090,947, the content of each of which is hereby incorporated herein by reference.

[0065] Fluorescent nucleotide sequences may also be used to label the sperm cells. Such nucleotide sequences fluoresce when hybridized to a nucleic acid containing a

target or complementary sequence, but are otherwise non-fluorescent when in a non-hybridized state. Such oligonucleotides are disclosed, for example, in U.S. Patent Application Publication No. 2003/0113765 (hereby incorporated herein by reference).

[0066] Sex specific antibodies may also be used to label the sperm cells in a staining mixture. In this embodiment, for example, a sex specific antibody may be conjugated with a fluorescent moiety (or equivalent reporter molecule). Because the antibody binds to antigens present on only an X chromosome-bearing or, alternatively, a Y chromosome-bearing cell, such cells can be selectively identified based upon their fluorescence (versus the non-fluorescence of an unlabeled cell). Moreover, more than one sex specific antibody, each antibody having a different fluorescent moiety attached thereto, may be used simultaneously. This allows for differentiation of X chromosome-bearing and Y chromosome-bearing cells based upon the differing fluorescence of each.

[0067] Luminescent, color-selective nanocrystals may also be used to label sperm cells in a staining mixture. Also referred to as quantum dots, these particles are well known in the art, as demonstrated by U.S. Patent No. 6,322,901 and U.S. Patent No. 6,576,291, each of which is hereby incorporated herein by reference. These nanocrystals have been conjugated to a number of biological materials, including for example, peptides, antibodies, nucleic acids, streptavidin, and polysaccharides, (see, for example, U.S. Patent Nos. 6,207,392; 6,423,551; 5,990,479, and 6,326,144, each of which is hereby incorporated herein by reference), and have been used to detect biological targets (see, for example, U.S. Patent Nos. 6,207,392 and

6,247,323, each of which is hereby incorporated herein by reference).

[0068] The preferred concentration of the DNA selective or of any other type of dye in the staining mixture is a function of a range of variables which include the permeability of the cells to the selected dye, the temperature of the staining mixture, the amount of time allowed for staining to occur, and the degree of enrichment desired in the subsequent sorting step. In general, the dye concentration is preferably sufficient to achieve the desired degree of staining in a reasonably short period of time without substantially detrimentally affecting sperm viability. For example, the concentration of Hoechst 33342, Hoechst 33258, SYBR-14, or BBC in the staining mixture will generally be between about 0.1μM and about 1.0M, preferably from about 0.1μM to about 700μM, and more preferably from about 100μM to about 200μM. In a particularly preferred embodiment, the concentration of Hoechst 33342, Hoechst 33258, SYBR-14, or BBC in the staining mixture will generally be between about 400μM to about 500μM, and most preferably about 450μM. Accordingly, under one set of staining conditions, the concentration of Hoechst 33342 is preferably about 100μM. Under another set of staining conditions, the concentration of Hoechst 33342 is about 150μM. Under still another set of staining conditions the concentration is preferably about 200μM. Under yet another set of staining conditions the concentration of Hoechst 33342 is most preferably about 450μM.

[0069] As another example, the concentration of a fluorescent polyamide, such as for example, those described in U.S. Application Publication No. 2001/0002314, will generally be between about 0.1μM and about 1mM, preferably

from about 1μM to about 1mM, more preferably about 5μM to about 100μM, even more preferably about 10μM.

[0070] Optionally, the staining mixture may also contain additives to enhance sperm viability. Exemplary additives include an antibiotic, a growth factor or a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly as discussed above with respect to cell sample collection. These additives may be added to the collection fluid in accordance therewith.

[0071] Once formed, the staining mixture may be maintained at any of a range of temperatures; typically, this will be within a range of about 4°C to about 50°C. For example, the staining mixture may be maintained at a "relatively low" temperature, *i.e.*, a temperature of about 4°C to about 30°C; in this embodiment, the temperature is preferably from about 20°C to about 30°C, more preferably from about 25°C to about 30°C, and most preferable at about 28°C. Alternatively, the staining mixture may be maintained within an "intermediate" temperature range, *i.e.*, a temperature of about 30°C to about 39°C; in this embodiment, the temperature is preferably at about 34°C to about 39°C, and more preferably about 37°C. In addition, the staining mixture may be maintained within a "relatively high" temperature range, *i.e.*, a temperature of about 40°C to about 50°C; in this embodiment, the temperature is preferably from about 40°C to about 45°C, more preferably from about 40°C to about 43°C, and most preferably at about 41°C. Selection of a preferred temperature generally depends upon a range of variables, including for example, the permeability of the cells to the dye(s) being used, the concentration of the dye(s) in the staining mixture, the amount of time the cells will be maintained in the staining

mixture, and the degree of enrichment desired in the sorting step.

[0072] Uptake of dye by the sperm cells in the staining mixture is allowed to continue for a period of time sufficient to obtain the desired degree of DNA staining. That period is typically a period sufficient for the dye to bind to the DNA of the sperm cells such that X and Y chromosome-bearing sperm cells may be sorted based upon the differing and measurable fluorescence intensity between the two. Generally, this will be no more than about 160 minutes, preferably no more than about 90 minutes, still more preferably no more than about 60 minutes, and most preferably from about 5 minutes to about 40 minutes.

[0073] Accordingly, in one embodiment, a staining mixture is formed comprising sperm cells, a motility inhibitor, and a dye in a concentration from about 100μM to about 200μM, and the staining mixture is held for a period of time at a temperature of about 41°C. In another embodiment, the motility inhibitor comprises 0.204g $NaHCO_3$, 0.433g $KHCO_3$, and 0.473g $C_6H_8O_7 \cdot H_2O$ per 25mL of purified water (0.097 moles/L of $NaHCO_3$, 0.173 moles/L of $KHCO_3$, 0.090 moles/L $C_6H_8O_7 \cdot H_2O$ in water).

[0074] In another embodiment, a staining mixture is formed comprising sperm cells, a motility inhibitor, and a dye in a concentration of about 400μM to about 500μM, and the staining mixture is held for a period of time at a temperature of about 41°C. In another embodiment, the dye concentration is 450μM. In another embodiment, the motility inhibitor comprises 0.204g $NaHCO_3$, 0.433g $KHCO_3$, and 0.473g $C_6H_8O_7 \cdot H_2O$ per 25mL of purified water (0.097 moles/L of $NaHCO_3$, 0.173 moles/L of $KHCO_3$, 0.090 moles/L $C_6H_8O_7 \cdot H_2O$ in water).

[0075] In still another embodiment, a staining mixture is formed comprising sperm cells, a motility inhibitor, and a dye in a concentration from about 100μM to about 200μM, and the staining mixture is held for a period of time at a temperature of about 28°C. In another embodiment, the motility inhibitor comprises 0.204g $NaHCO_3$, 0.433g $KHCO_3$, and 0.473g $C_6H_8O_7 \cdot H_2O$ per 25mL of purified water (0.097 moles/L of $NaHCO_3$, 0.173 moles/L of $KHCO_3$, 0.090 moles/L $C_6H_8O_7 \cdot H_2O$ in water).

[0076] In yet another embodiment, a staining mixture is formed comprising sperm cells, a motility inhibitor, and a dye in a concentration from about 400μM to about 500μM, and the staining mixture is held for a period of time at a temperature of about 28°C. In another embodiment, the dye concentration is 450μM. In another embodiment, the motility inhibitor comprises 0.204g $NaHCO_3$, 0.433g $KHCO_3$, and 0.473g $C_6H_8O_7 \cdot H_2O$ per 25mL of purified water (0.097 moles/L of $NaHCO_3$, 0.173 moles/L of $KHCO_3$, 0.090 moles/L $C_6H_8O_7 \cdot H_2O$ in water).

Sorting

[0077] A motility inhibitor may also be used to render the sperm cells immotile during sorting of the sperm cells. Generally, once the sperm are stained according to the present invention, they may be sorted according to any known means that allows for separation based upon fluorescence. Commonly used and well known methods include flow cytometry systems, as exemplified by and described in U.S. Patent Nos. 5,135,759, 5,985,216, 6,071,689, 6,149,867, and 6,263,745, International Patent Publications WO 99/33956 and WO 01/37655, and U.S. Patent Application Serial No. 10/812,351, the content of which is hereby incorporated herein by reference, and corresponding

International Patent Publication WO 2004/088283. When sorting according to such methods, the sperm are introduced into the nozzle of a flow cytometer in a sample fluid. In one embodiment, therefore, the sample fluid may comprise the stained sperm cells and a motility inhibitor.

[0078] Likewise, the sheath fluid used to surround the stream of sample fluid as it travels through the cytometer may also comprise a motility inhibitor. Generally, the sheath fluid may be introduced into a nozzle of the cytometer using pressurized gas or by a syringe pump. Preferably, the pressurized gas is carbon dioxide or nitrogen, more preferably nitrogen. Alternatively, the pressurized gas may be carbon dioxide, although under such circumstances, care may be taken to minimize effervescence.

[0079] Optionally, the sample fluid or sheath fluid may also contain additive, such as, an antibiotic, a composition which regulates oxidation/reduction reactions intracellularly or extracellularly, or a growth factor as discussed above with respect to cell sample collection Each of these additives may be added to either fluid in accordance therewith.

Collection of the sorted cells

[0080] Once sorted, the sorted cells are collected in a vessel that contains a collection fluid. Generally, the purpose of the collection fluid includes cushioning the impact of the sperm cells with the collection vessel or providing a fluid support for the cells.

[0081] In one embodiment, the collection fluid comprises a motility inhibitor and a protein source. If included, the protein source may be any protein source that does not interfere with the viability of the sperm cells and is compatible with the motility inhibitor. Examples of

common protein sources include milk (including heat homogenized and skim), milk extract, egg yolk, egg yolk extract, soy protein and soy protein extract. Such proteins may be used in a concentration from about 1% (v/v) to about 30% (v/v), preferably from about 10% (v/v) to about 20% (v/v), and more preferably about 10% (v/v).

[0082] Optionally, the collection fluid may also contain additives such as, an antibiotic, a growth factor or a composition which regulates oxidation/reduction reactions intracellularly or extracellularly as discussed above with respect to cell sample collection. Each of these additives may be added to the collection fluid in accordance therewith.

[0083] Accordingly, in a certain embodiment, the collection fluid comprises 0.097 moles/L of $NaHCO_3$, 0.173 moles/L of $KHCO_3$, 0.090 moles/L $C_6H_8O_7 \cdot H_2O$ and 10% (v/v) egg yolk in water, at a pH of about 6.2, more preferably of about 7.0, and even more preferably of about 6.5. Preferably, the collection fluid is maintained under an atmosphere having an enriched partial pressure of carbon dioxide relative to air; for example, the atmosphere may have a partial pressure of carbon dioxide in excess of 0.9, more preferably 0.95 and still more preferably 0.99.

[0084] In lieu of the use of a more traditional collection fluid, the sorted cells may be collected into a vessel containing or coated with a cryoextender. Accordingly, in one particular embodiment, the sorted cells are collected into a cryoextender comprising a motility inhibitor. In another embodiment, the sorted cells are collected into a cryoextender comprising a motility inhibitor, water, Triladyl® (Minitube, Verona, WI, comprising glycerol, tris, citric acid, fructose, 5mg/100ml tylosin, 25mg/100ml gentamycin, 30mg/100ml Spectinomycin,

and 15mg/100ml Lincomycin), egg yolk, and pyruvic acid. In yet another embodiment, the collection fluid is the cryoextender comprising 0.097 moles/L of $NaHCO_3$, 0.173 moles/L of $KHCO_3$, 0.090 moles/L $C_6H_8O_7 \cdot H_2O$ in water, and 25g Triladyl®, 25g egg yolk, and 10mM pyruvic acid per 75mL of water.

Cryoextension of the sorted cells

[0085] Once the sperm have been sorted and collected into collection vessels, they may be used for inseminating female mammals. This can occur almost immediately, requiring little additional treatment of the sperm. Likewise, the sperm may also be cooled or frozen for use at a later date. In such instances, the sperm may benefit from the addition of a cryoextender to minimize the impact upon viability or post-thaw motility as a result of cooling and freezing.

[0086] A motility inhibitor may be used to render cells in the cryoextender immotile. Generally, a cryoextender may comprise a motility inhibitor, a protein source, and a cryoprotectant. If included, a protein source may be added to provide support to the cells and to cushion the contact of the cells with the collection vessel. The protein source may be any protein source that does not interfere with the viability of the sperm cells and is compatible with the motility inhibitor. Examples of common protein sources include milk (including heat homogenized and skim), milk extract, egg yolk, egg yolk extract, soy protein and soy protein extract. Such proteins may be found in a concentration from about 10% (v/v) to about 30% (v/v), preferably from about 10% (v/v) to about 20% (v/v), and more preferably about 20% (v/v).

[0087] A cryoprotectant is preferably included in the cryoextender to lessen or prevent cold shock or to maintain fertility of the sperm. Numerous cryoprotectants are known in the art. Selection of a cryoprotectant suitable for use with a given extender may vary, and depends upon the species from which the sperm to be frozen were obtained. Examples of suitable cryoprotectants include, for example, glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, trehalose, Triladyl®, and combinations thereof. If included, generally, these cryoprotectants are present in the cryoextender in an amount of about 1% (v/v) to about 15% (v/v), preferably in an amount of about 5% (v/v) to about 10% (v/v), more preferably in an amount of about 7% (v/v), and most preferably in an amount of about 6% (v/v).

[0088] In one particular embodiment, the cryoextender comprises a motility inhibitor, water, Triladyl®, egg yolk, and pyruvic acid. In yet another embodiment, the cryoextender comprises 0.097 moles/L of $NaHCO_3$, 0.173 moles/L of $KHCO_3$, 0.090 moles/L $C_6H_8O_7 \cdot H_2O$ in water, and 25g Triladyl®, 25g egg yolk, and 10mM pyruvic acid per 75mL of water.

[0089] In another particular embodiment, the cryoextender comprises a motility inhibitor, water, Triladyl®, and egg yolk. In yet another embodiment, the cryoextender comprises 0.097 moles/L of $NaHCO_3$, 0.173 moles/L of $KHCO_3$, 0.090 moles/L $C_6H_8O_7 \cdot H_2O$ in water, and 25g Triladyl®, and 25g egg yolk per 75mL of water.

[0090] Optionally, the cryoextender may also contain an antibiotic, a growth factor or a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly as discussed above with respect to

cell sample collection Each of these additives may be added to the collection fluid in accordance therewith.

[0091] Having described the invention in detail, it will be apparent that modifications and variations are possible without departing the scope of the invention defined in the appended claims.

EXAMPLES

[0092] The following non-limiting examples are provided to further illustrate the present invention.

Example 1

[0093] Bull semen was collected from a sexually mature bull using an artificial vagina and transported at 25°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. *Theriogenology*, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, several tubes of 150 X 10^6 sperm/ml suspensions were prepared by suspending semen in a TCA buffer or a carbonate-based inhibitor. Table I. below illustrates the compositions and staining conditions used.

Table I.

Sample Name	Composition	pH	Conc. (uM) Hoechst	Temperature (°C)
10mM pyr TCA	10mM pyruvate in TCA	7.3	600μM	28°C
10mM pyr CO_2	10mM pyruvate in TCA blanket with CO_2 balloon	7.3	600μM	28°C
Carbonate 6.2	Carbonate based inhibitor, pH 6.2	6.2	600μM	28°C
Carbonate 7.3	Carbonate based inhibitor, pH 7.3	7.3	600μM	28°C

[0094] To the sperm suspensions, aliquots of a 10mM Hoechst solution in water were added to yield a concentration of 600μM Hoechst. The sperm suspensions were maintained in a 28°C water bath for the duration of the staining period (approximately 1 hour). Sperm suspensions were analyzed by removing a 50μL aliquot from the stained sperm suspension, adding 200μL of 25°C 10mM pyruvate in TCA at pH 7.3 to initiate the reversal of the quiescence, allowing at least a five minute equilibration period, and analyzing by IVOS to measure percent progressive motility (% Prog. Mot.). Comparisons of the IVOS percent progressive motilities are seen in Figures 1-3.

Example 2

[0095] Bull semen was collected from a sexually mature bull using an artificial vagina and transported at 25°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. Theriogenology, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, several tubes of 450 X 10^6 sperm/ml suspensions were prepared by suspending semen in either a TCA buffer or a carbonate based inhibitor. Table II. below illustrates the compositions and staining conditions used.

Table II.

Sample Name	Composition	pH	Conc. (uM) Hoechst	Temperature (°C)
10mM pyr TCA	10mM pyruvate in TCA	7.3	1000μM	28°C
Carbonate 6.2	Carbonate based inhibitor, pH 6.2	6.2	1000μM	28°C
Carbonate 7.3	Carbonate based inhibitor, pH 7.3	7.3	1000μM	28°C

[0096] To the sperm suspensions, aliquots of a 10mM Hoechst solution in water were added to yield a concentration of 1000μM Hoechst. The sperm suspensions were maintained in a 28°C water bath for 1 hour, and were then diluted to 150 X 10^6 sperm/ml with 10mM pyruvate in TCA or a carbonate-based inhibitor at a pH 6.2 as specifically indicated in each figure to dilute to a concentration

typical for sorting. Sperm suspensions were analyzed by removing a 50μL aliquot from the stained and diluted sperm suspension at the time period designated within each figure and adding 200μL of 25°C 10mM pyruvate in TCA at pH 7.3 to initiate the reversal of the quiescence, allowing at least a five minute equilibration period, and analyzing the aliquot by IVOS to measure the percent progressive motility. Comparisons of the IVOS percent progressive motilities are seen in Figures 4-6.

Example 3

[0097] Bull semen was collected from a sexually mature bull using an artificial vagina and transported at 25°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. Theriogenology, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, several tubes of 450 X 10^6 sperm/ml suspensions were prepared by suspending semen in either a TCA buffer or a carbonate based inhibitor. Table II. below illustrates the compositions and staining conditions used.

Table III

Sample Name	Buffer	pH	Conc (uM) Hoechst	Temperature (°C)
10mM pyr TCA	10mM pyruvate in TCA	7.3	300μM	41°C
Carbonate 6.2	Carbonate based inhibitor, pH 6.2	6.2	300μM	41°C
Carbonate 7.3	Carbonate based inhibitor, pH 7.3	7.3	300μM	41°C

[0098] To the sperm suspensions, aliquots of a 10mM Hoechst solution in water were added to yield a concentration of 300μM Hoechst. The sperm suspensions were maintained in a 41°C water bath for 30 minutes, and then diluted to 150 X 10^6 sperm/ml with 10 mM pyruvate in TCA or a carbonate-based inhibitor at pH 6.2 as specifically indicated in each figure to dilute to a concentration typical for sorting. Sperm suspensions were analyzed by removing a 50μL aliquot from the stained and diluted sperm suspension at the time period designated within each figure and adding 200μL of 25°C 10mM pyruvate in TCA at pH 7.3 to initiate the reversal of the quiescence, allowing at least a five minute equilibration period, and analyzing by IVOS to measure the percent progressive motility. Comparisons of the IVOS percent progressive motilities are seen in Figures 7-9.

Example 4

[0099] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample diluted in 2 parts carbonate buffer for transportation at 25°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. *Theriogenology*, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, 1mL of 150 X 10^6 sperm/ml suspension was prepared by removing an aliquot of the carbonate sperm suspension centrifuging the sperm suspension at 500 X g for 5 minutes, removing the supernatant and re-suspending the pellet in 41°C TCA buffer pH 7.3. An additional 1mL of 150 X 10^6 sperm/ml was

prepared by suspending an aliquot of semen in 41°C TCA buffer containing 10mM pyruvate at pH 7.3. To the sperm suspensions, aliquots of a 10mM Hoechst solution in water were added to yield the dye concentration of 400μM Hoechst. The sperm suspensions were maintained in a 41°C water bath for the duration of the staining period. Sperm suspensions were analyzed by removing a 50μL aliquot from the staining sperm suspension, adding 200μL of the same buffer at the same temperature and analyzing by IVOS to measure % progressive motility (% Prog Mot). Results of the IVOS analysis are summarized in Figure 10.

Example 5

[00100] Sperm samples were obtained and prepared in the same manner as in Example 4 with the following exception. The buffer used to suspend the sperm for staining and IVOS analysis were TCA and TCA containing 10uM Vitamin K. Results of the IVOS analysis are summarized in Figure 11.

Example 6

[00101] Sperm samples were obtained and prepared in the same manner as in Example 4 with the following exception. The buffer used to suspend the sperm for staining and IVOS analysis were TCA and TCA containing 100uM Vitamin K. Results of the IVOS analysis are summarized in Figure 12.

Example 7

[00102] Sperm samples were obtained and prepared in the same manner as in Example 4 with the following exception. The buffers used to suspend the sperm for staining and IVOS analysis were TCA and TCA containing 1mM Lipoic Acid. Results of the IVOS analysis are summarized in Figure 13.

Example 8

[00103] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample diluted in 2 parts carbonate buffer for transportation at 25°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. *Theriogenology*, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, 1mL of 150 X 10^6 sperm/ml suspension was prepared by centrifuging the sperm suspension at 500 X g for 5 minutes, removing the supernatant and re-suspending the pellet in 28°C TCA buffer pH 7.3. An additional 1mL of 150 X 10^6 sperm/ml was prepared by suspending an aliquot of semen in 28°C TCA buffer containing 10mM pyruvate at pH 7.3. To the sperm suspensions, aliquots of a 10 mM Hoechst solution in water were added to yield the dye concentration of 600µM Hoechst. The sperm suspensions were maintained in 28°C water bath for the duration of the staining period. Sperm suspensions were analyzed by removing a 50µL aliquot from the staining sperm suspension, adding 200µL of the same buffer at the same temperature and analyzing by IVOS to measure percent progressive motility (% Prog Mot). Results of the IVOS analysis are summarized in Figure 14.

Example 9

[00104] Sperm samples were obtained and prepared in the same manner as in Example 8 with the following exception. The buffer used to suspend the sperm for staining and IVOS

analysis were TCA and TCA containing 100uM Vitamin K. Results of the IVOS analysis are summarized in Figure 15.

Example 10

[00105] Sperm samples were obtained and prepared in the same manner as in Example 8 with the following exception. The buffer used to suspend the sperm for staining and IVOS analysis were TCA and TCA containing 1mM Lipoic Acid. Results of the IVOS analysis are summarized in Figure 16.

Example 11

[00106] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample diluted in 2 parts carbonate buffer for transportation at 25°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. *Theriogenology*, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, 1mL of 150 X 10^6 sperm/ml suspensions were prepared by removing an aliquots of the carbonate sperm suspension, centrifuging the sperm suspension at 500 X g for 5 minutes, removing the supernatant and re-suspending the pellet in 1 ml TCA buffer or in 1 ml TCA buffer with 2.5 mM, 10 mM, 25 mM, or 50 mM pyruvate. To the samples was added MON33342 solution to yield the final dye concentrations of 600µM. The suspensions were incubated in a 28°C water bath. Stained sperm suspensions were analyzed by removing a 50µL aliquot from the staining sperm suspension, adding 200µL of the same buffer at the same temperature and analyzing by IVOS

to measure percent progressive motility (% Prog Mot). IVOS results for % Prog Mot are shown in Figures 17.

Example 12

[00107] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample diluted in 2 parts carbonate buffer for transportation at 25°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. *Theriogenology*, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, 1mL of 150 X 10^6 sperm/ml suspension in TCA buffer was prepared by removing an aliquot of the carbonate sperm suspension, centrifuging the sperm suspension at 500 X g for 5 minutes, removing the supernatant and re-suspending the pellet in 1mL TCA buffer. 1ml of 150 X 10^6 sperm/ml suspension in 10mM pyruvate in TCA was prepared by removing an aliquot of the carbonate sperm suspension, centrifuging the sperm suspension at 500 X g for 5 minutes, removing the supernatant and re-suspending the pellet in 1mL of 10 mM pyruvate TCA buffer. To samples was added SYBR 14 dye solution to yield the final dye concentrations of 20μM The suspensions were incubated in a 28°C water bath. Sperm suspensions were analyzed by removing a 50μL aliquot from the staining sperm suspension, adding 200μL of the same buffer at the same temperature and analyzing by IVOS to measure percent progressive motility (% Prog Mot). IVOS results for % Prog Mot are shown in Figures 18.

Example 13

[00108] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample diluted in 2 parts carbonate buffer for transportation at 25°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. *Theriogenology*, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, 1mL of 150 X 10^6 sperm/ml suspension in TCA buffer was prepared by removing an aliquot of the carbonate sperm suspension, centrifuging the sperm suspension at 500 X g for 5 minutes, removing the supernatant and re-suspending the pellet in 1mL TCA buffer. 1ml of 150 X 10^6 sperm/ml suspension in 10mM pyruvate in TCA was prepared by removing an aliquot of the carbonate sperm suspension, centrifuging the sperm suspension at 500 X g for 5 minutes, removing the supernatant and re-suspending the pellet in 1ml of 10 mM pyruvate TCA buffer. To the samples was added BBC solution to yield the final dye concentrations of 100μM. The suspensions were incubated in a 28°C water bath. Stained sperm suspensions were analyzed by removing a 50μL aliquot from the staining sperm suspension, adding 200μL of the same buffer at the same temperature and analyzing by IVOS to measure percent progressive motility (% Prog Mot). IVOS results for % Prog Mot are shown in Figures 19.

Example 14

[00109] Sperm samples were obtained and prepared in the same manner as in Example 4 with the following exception. The staining concentration was 200uM BBC. Results of the IVOS analysis are summarized in Figure 20.

WHAT IS CLAIMED IS:

1. A sperm cell suspension comprising viable spermatozoa and a composition which down-regulates carbohydrate uptake by the spermatozoa, the concentration of spermatozoa in the suspension being less than about 1 X 10^6 or at least about 1 x 10^8 spermatozoa per ml.

2. A sperm cell suspension comprising viable, immotile sperm, the concentration of spermatozoa in the suspension being less than about 1 X 10^6 or at least about 1 x 10^8 spermatozoa per ml.

3. A sperm cell suspension comprising viable spermatozoa, the spermatozoa having a motility more characteristic of epididymal spermatozoa than endogenous ejaculated spermatozoa of the same species, the concentration of spermatozoa in the suspension being less than about 1 X 10^6 or at least about 1 x 10^8 spermatozoa per ml.

4. A sperm cell suspension comprising viable sperm, potassium and optionally sodium, the concentration of spermatozoa in the suspension being less than about 1 X 10^6 or at least about 1 x 10^8 spermatozoa per ml and the molar ratio of potassium to sodium being greater than 1:1, respectively.

5. The suspension of claim 4 wherein the molar ratio of potassium to sodium is greater than 1.25:1, respectively.

6. The suspension of claim 4 wherein the molar ratio of potassium to sodium is greater than 1.5:1, respectively.

7. The suspension of claim 4 wherein the molar ratio of potassium to sodium is greater than 1.75:1, respectively.

8. The suspension of claim 4 wherein the molar ratio of potassium to sodium is greater than 2:1, respectively.

9. A sperm cell suspension comprising viable spermatozoa, a composition which down-regulates carbohydrate uptake by the spermatozoa, and a DNA-selective dye.

10. A sperm cell suspension comprising viable, immotile sperm and a DNA-selective dye.

11. A sperm cell suspension comprising viable spermatozoa and a DNA-selective dye, the spermatozoa having a metabolic rate and motility more characteristic of epididymal spermatozoa than endogenous ejaculated spermatozoa of the same species.

12. A sperm cell suspension comprising viable, immotile spermatozoa, the spermatozoa having a DNA-selective dye associated with their DNA.

13. A sperm cell suspension comprising viable spermatozoa, the spermatozoa having a metabolic rate and motility more characteristic of epididymal spermatozoa than endogenous ejaculated spermatozoa of the same species, the spermatozoa also having a DNA-selective dye associated with their DNA.

14. The suspension of claim 10, wherein the dye is a DNA selective fluorescent dye.

15. The suspension of claim 10, wherein the dye is a UV excitable or a visible light excitable dye.

16. The suspension of claim 10, wherein the dye is selected from the group consisting of Hoechst 33342, Hoechst 33258, SYBR-14, and bisbenzimide-BODIPY® conjugate 6-{[3-((2Z)-2-{[1-(difluoroboryl)-3,5-dimethyl-1*H*-pyrrol-2-yl]methylene}-2*H*-pyrrol-5-yl)propanoyl]amino}-*N*-[3-(methyl{3-[({4-[6-(4-methylpiperazin-1-yl)-1*H*,3'*H*-2,5'-bibenzimidazol-2'-yl]phenoxy}acetyl)amino]propyl}amino)propyl]hexanamide.

17. The sperm suspension of claim 2, wherein the suspension comprises a source of carbonate.

18. The sperm suspension of claim 2, wherein the suspension comprises $NaHCO_3$, $KHCO_3$, and $C_6H_8O_7 \cdot H_2O$.

19. The sperm suspension of claim 2, wherein the suspension is formed from a buffer comprising 0.097 moles/L of $NaHCO_3$, 0.173 moles/L of $KHCO_3$, 0.090 moles/L $C_6H_8O_7 \cdot H_2O$ in water.

20. The sperm suspension of claim 2, wherein the concentration of spermatozoa in the suspension is at least 1.25×10^8 spermatozoa per ml.

21. The sperm suspension of claim 2, wherein the concentration of spermatozoa in the suspension is at least 1.5×10^8 spermatozoa per ml.

22. The sperm suspension of claim 2, wherein the concentration of spermatozoa in the suspension is at least 1.75×10^8 spermatozoa per ml.

23. The suspension of claim 2, wherein the concentration of spermatozoa in the suspension is less than about 9.0×10^5 spermatozoa per ml.

24. The suspension of claim 2, wherein the concentration of spermatozoa in the suspension is less than about 7×10^5 spermatozoa per ml.

25. The suspension of claim 2, wherein the concentration of spermatozoa in the suspension is less than about 5×10^5 spermatozoa per ml.

26. The suspension of claim 2, wherein the concentration of spermatozoa in the suspension is less than about 2×10^5 spermatozoa per ml.

27. The suspension of claim 2, wherein the concentration of spermatozoa in the suspension is less than about 1×10^5 spermatozoa per ml.

28. A process for staining sperm cells, the process comprising forming a staining mixture containing intact viable sperm cells, a motility inhibiting amount of potassium, and a DNA selective dye.

29. The process of claim 28 wherein the staining mixture is under an atmosphere having an enriched partial pressure of CO_2 relative to air.

30. A process of forming a sperm cell suspension for use in cell sorting, the process comprising combining a sperm cell source with a composition which inhibits the motility of sperm cells to form a sperm cell suspension, the concentration of sperm cells in the suspension being less than about 1 X 10^6 or at least 1 x 10^8 sperm cells per milliliter.

31. A process of forming a sperm cell suspension for use in a flow cytometry process, the process comprising collecting the ejaculate of a mammal in a buffer containing an inhibitory amount of a motility inhibitor to form a sperm cell suspension, the suspension comprising less than about 1 X 10^6 or at least 1 x 10^8 sperm cells per milliliter.

32. The process of claim 30, wherein the cell suspension comprises a DNA-selective dye.

33. The suspension of claim 18, further comprising a DNA-selective dye.

FIG. 1

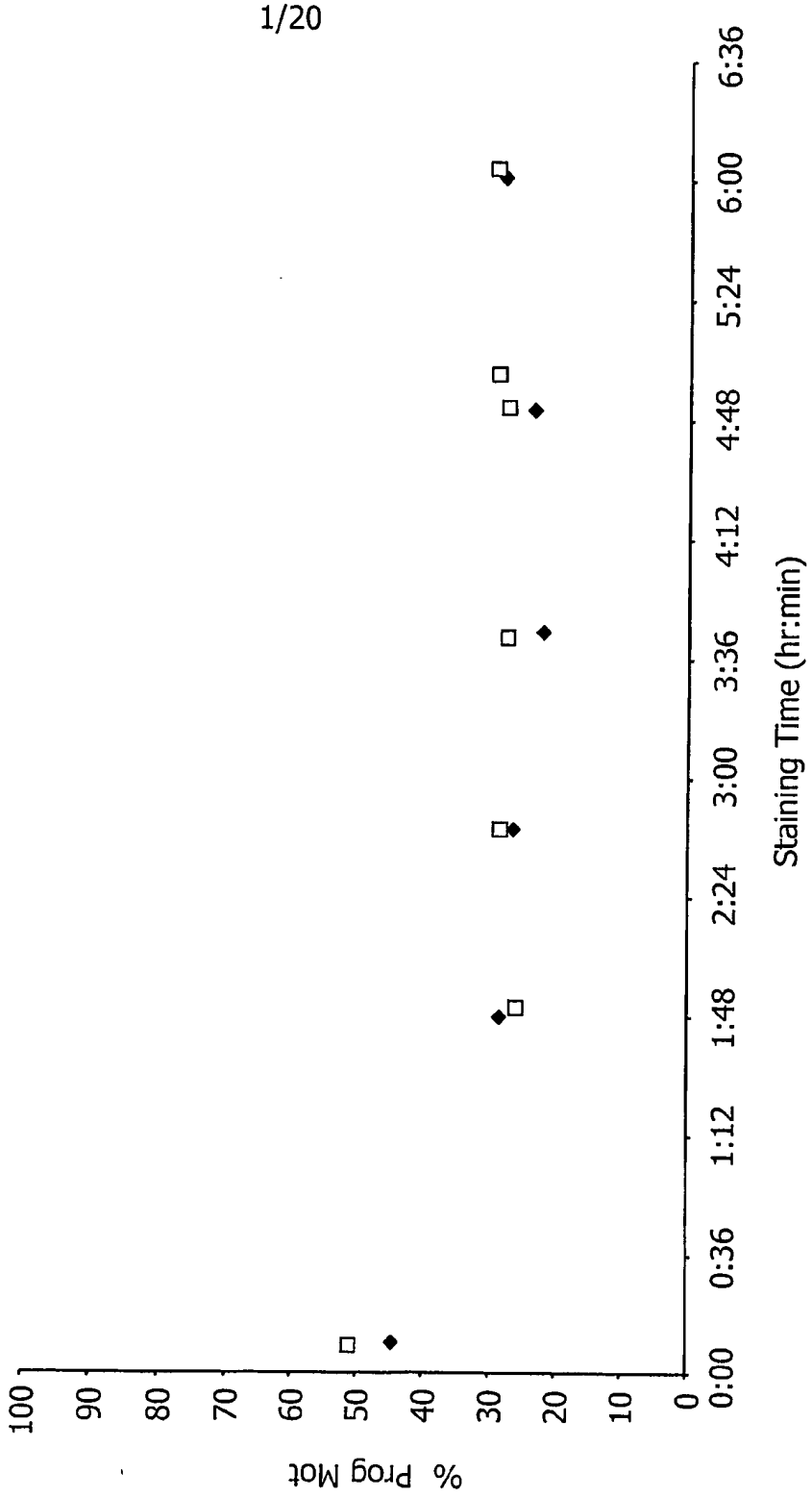

◆ 10 mM pyruvate in TCA □ 10 mM pyruvate in TCA under CO2
% Prog Mot
100
90
80
70
60
50
40
30
20
10
0
0:00
0:36
1:12
1:48
2:24
3:00
3:36
4:12
4:48
5:24
6:00
6:36
Staining Time (hr:min)

FIG. 2

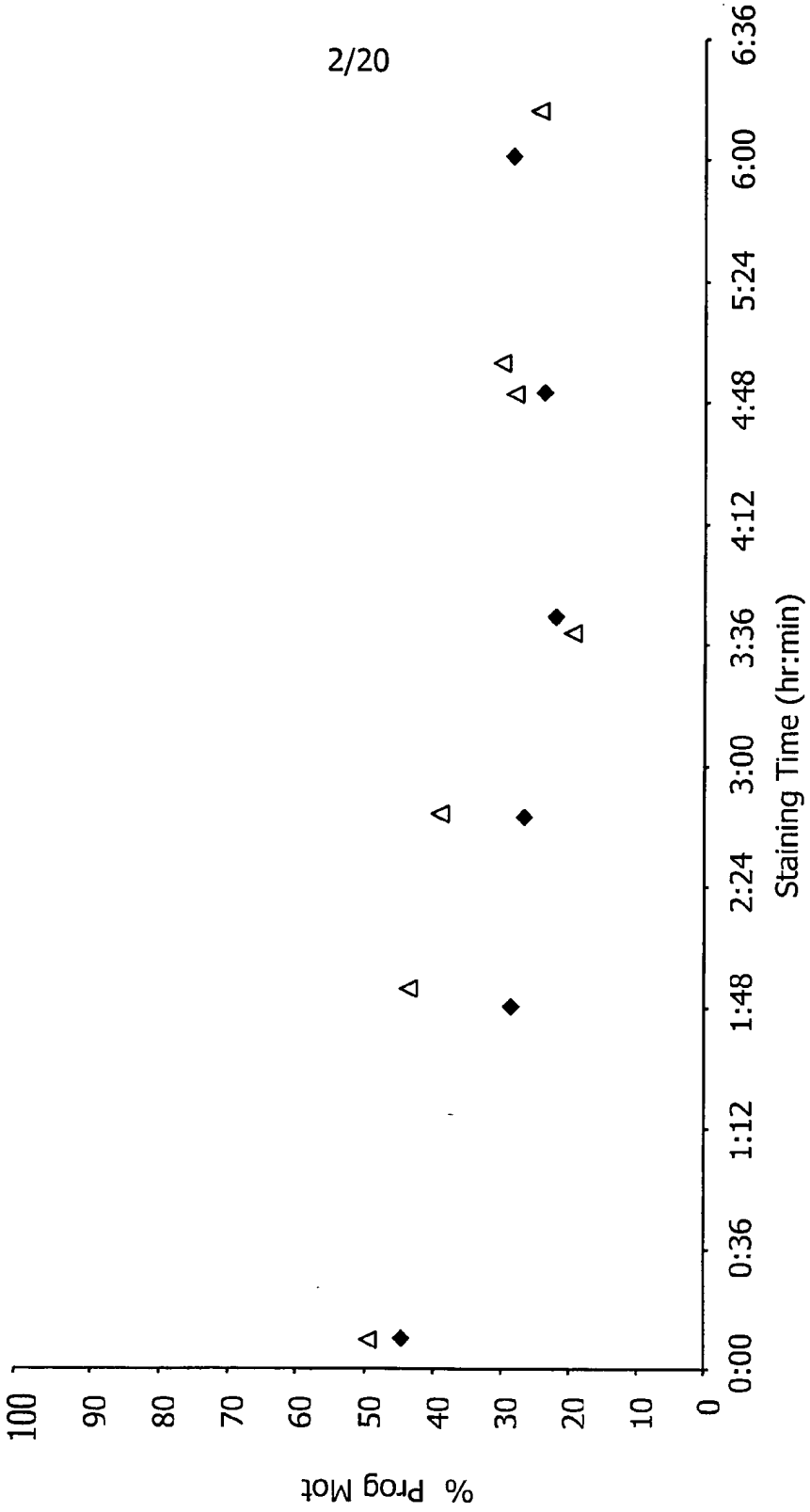

◆ 10 mM pyruvate in TCA
△ Carbonate buffer pH 7.3
% Prog Mot
100
90
80
70
60
50
40
30
20
10
0
0:00
0:36
1:12
1:48
2:24
3:00
3:36
4:12
4:48
5:24
6:00
6:36
Staining Time (hr:min)

FIG. 3

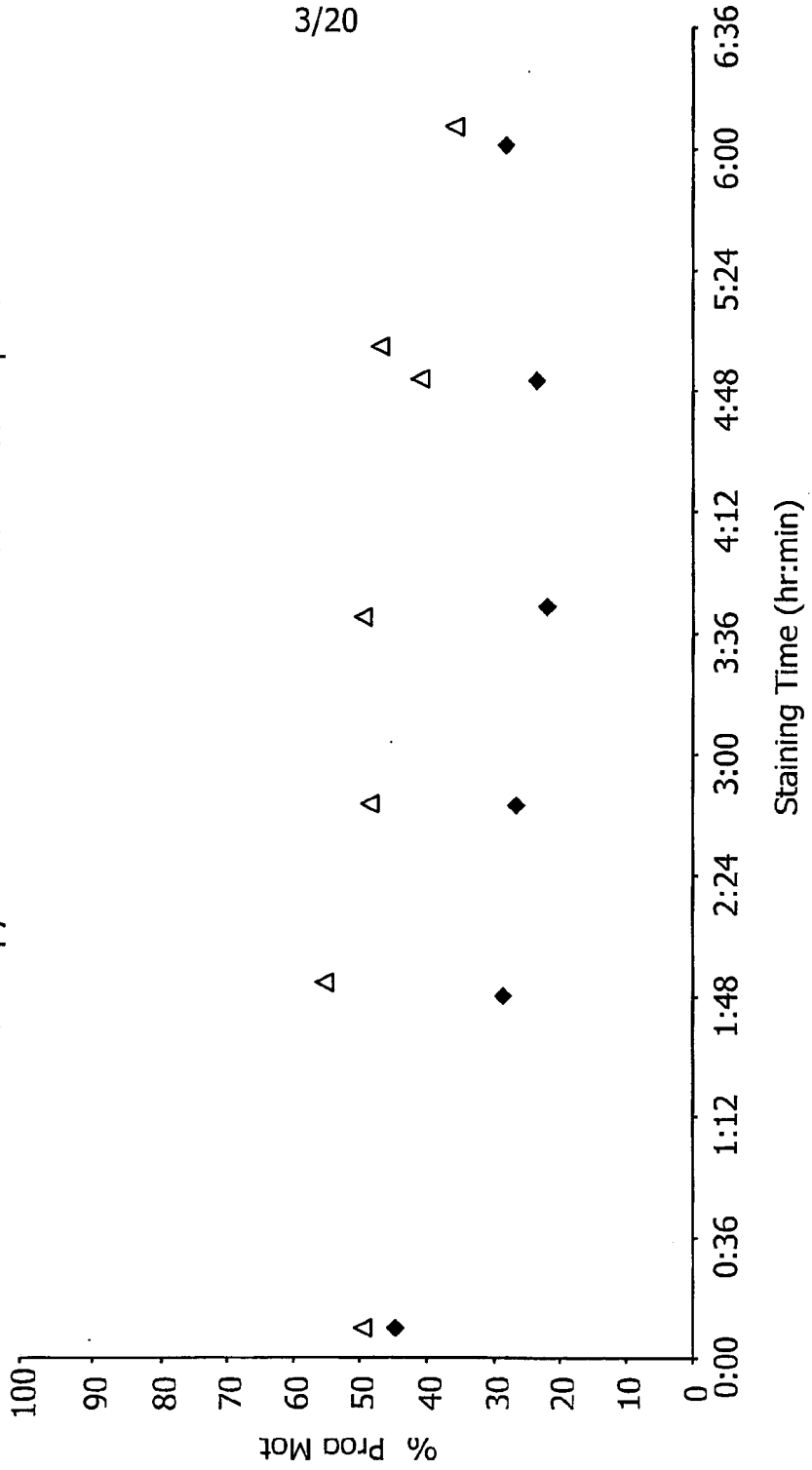

◆ 10 mM pyruvate in TCA
Δ Carbonate buffer pH 6.2
% Prod Mat
100
90
80
70
60
50
40
30
20
10
0
0:00
0:36
1:12
1:48
2:24
3:00
3:36
4:12
4:48
5:24
6:00
6:36
Staining Time (hr:min)

FIG. 4

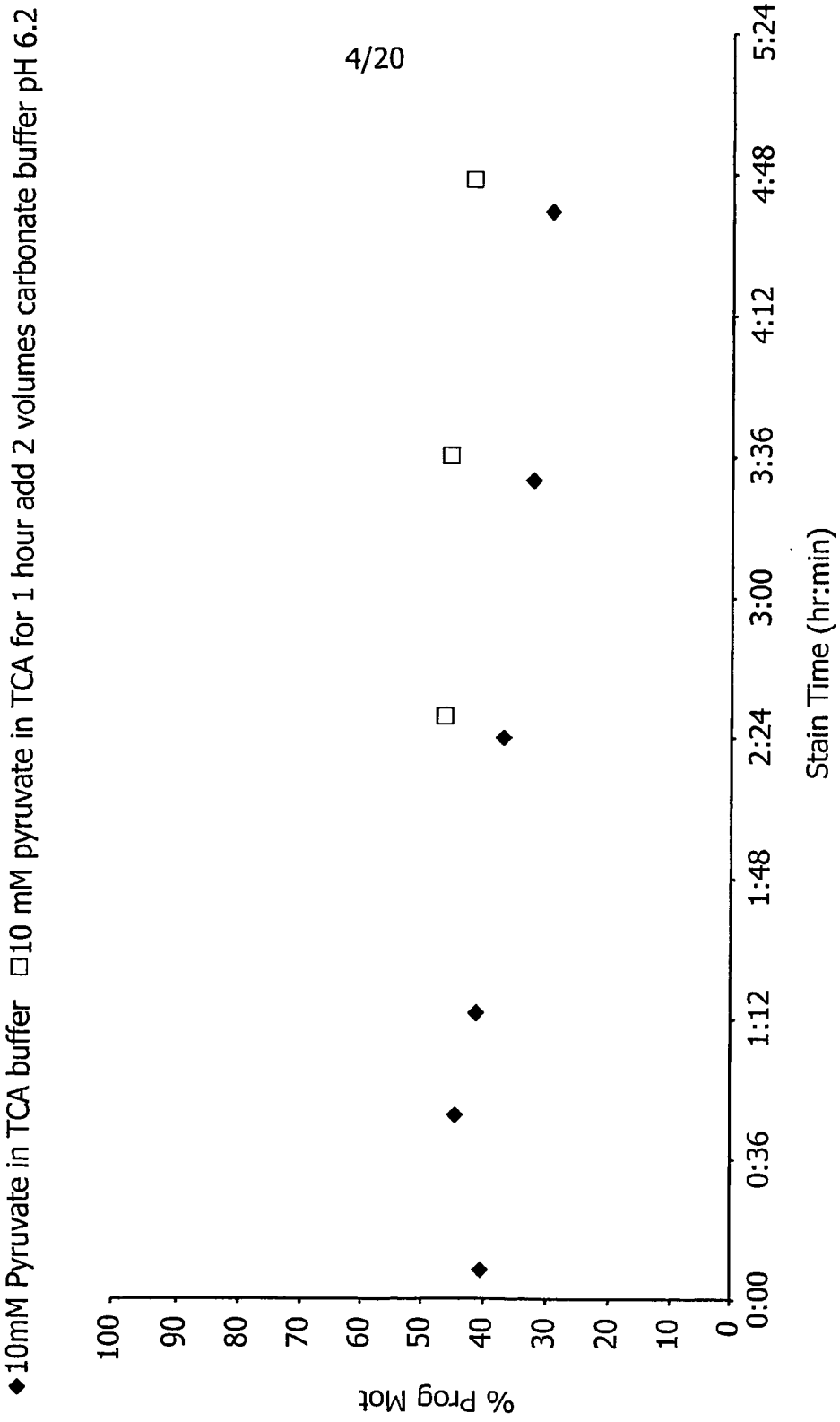

◆10mM Pyruvate in TCA buffer □10 mM pyruvate in TCA for 1 hour add 2 volumes carbonate buffer pH 6.2
% Prog Mot
100
90
80
70
60
50
40
30
20
10
0
0:00
0:36
1:12
1:48
2:24
3:00
3:36
4:12
4:48
5:24
Stain Time (hr:min)

FIG. 5

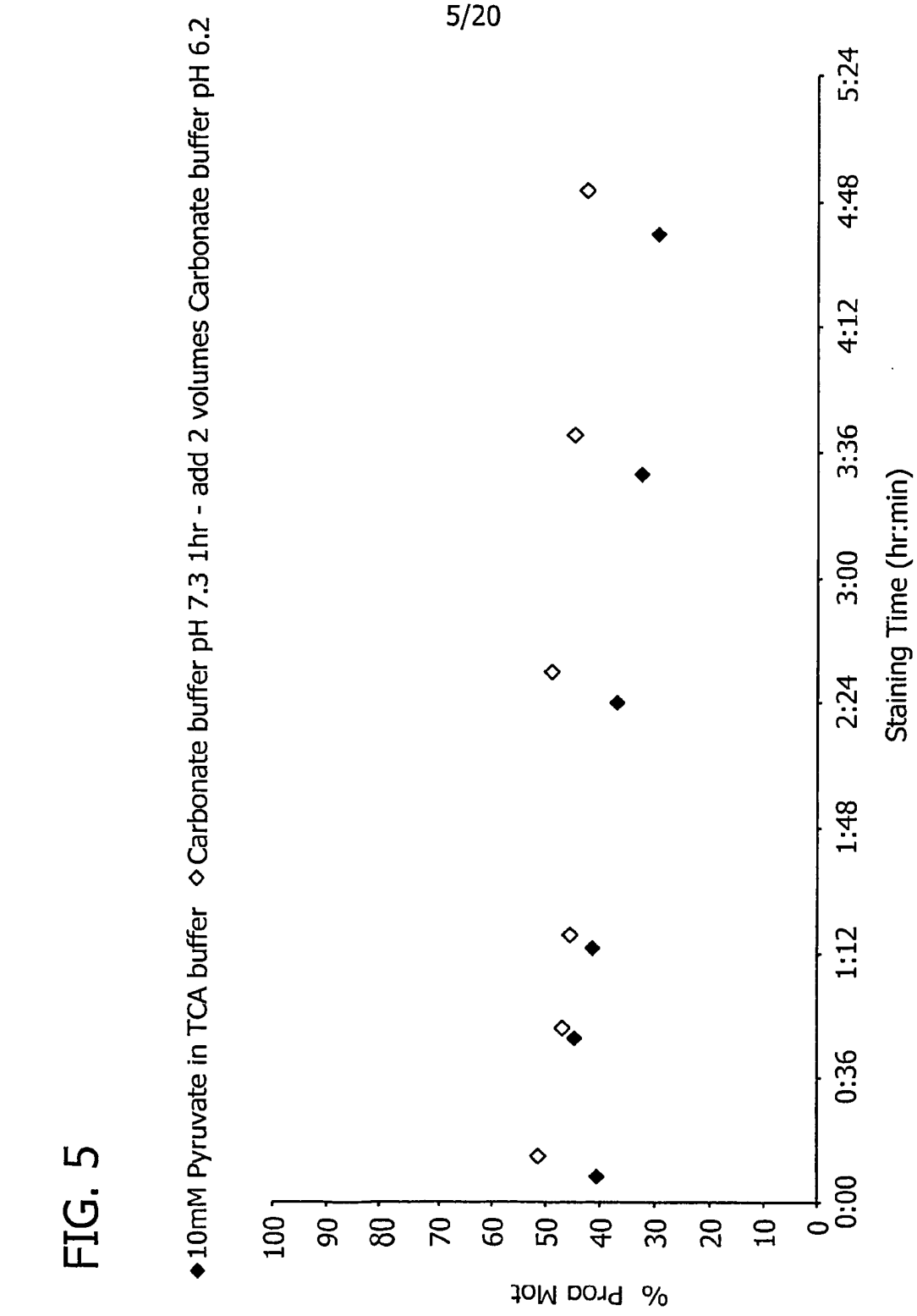

◆10mM Pyruvate in TCA buffer ◇Carbonate buffer pH 7.3 1hr - add 2 volumes Carbonate buffer pH 6.2
% Prod Mat
100
90
80
70
60
50
40
30
20
10
0
0:00
0:36
1:12
1:48
2:24
3:00
3:36
4:12
4:48
5:24
Staining Time (hr:min)

FIG. 6

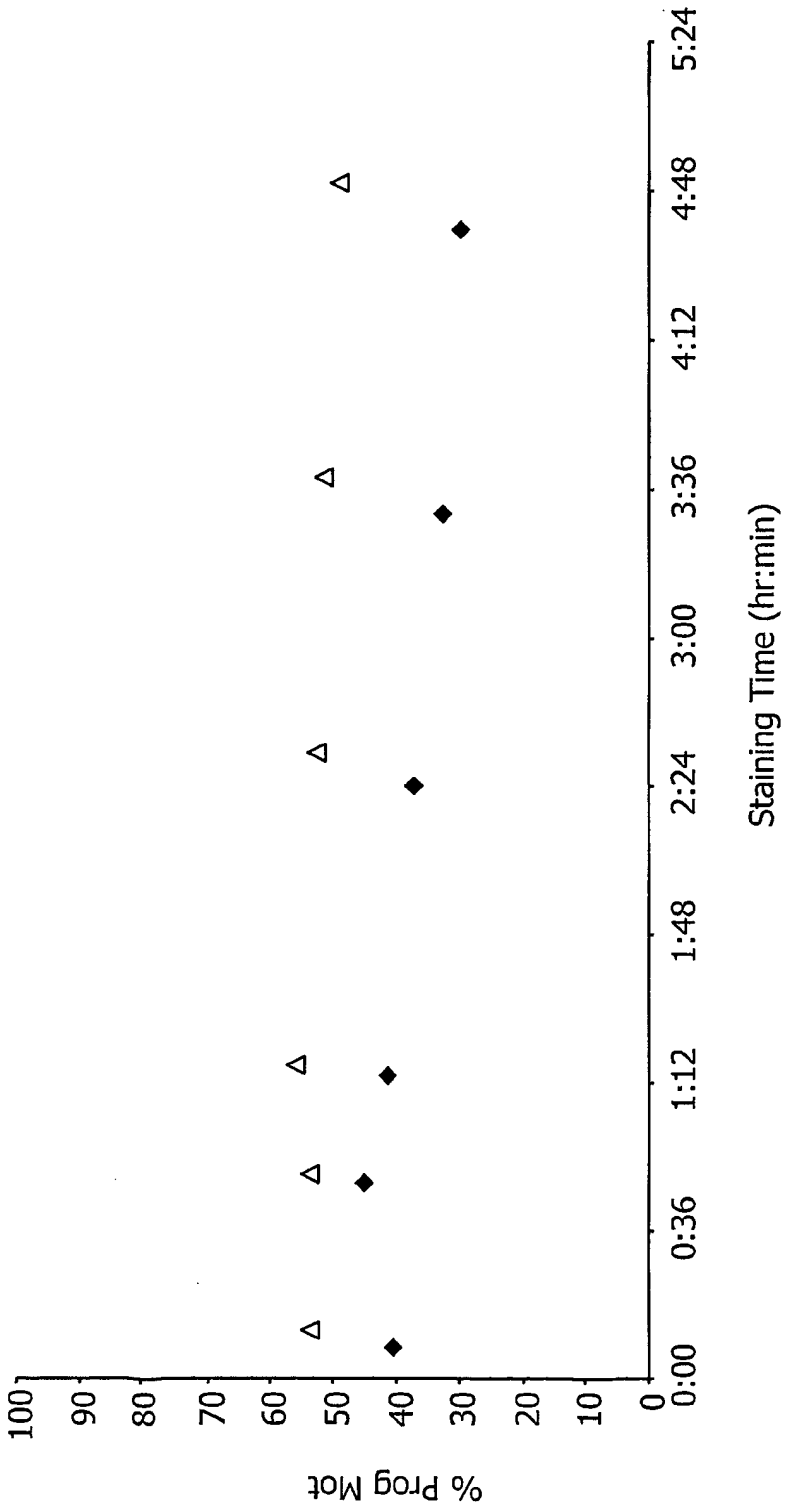

◆10mM Pyruvate in TCA buffer
Δ Carbonate buffer pH 6.2
% Prog Mot
100
90
80
70
60
50
40
30
20
10
0
0:00
0:36
1:12
1:48
2:24
3:00
3:36
4:12
4:48
5:24
Staining Time (hr:min)

FIG. 7

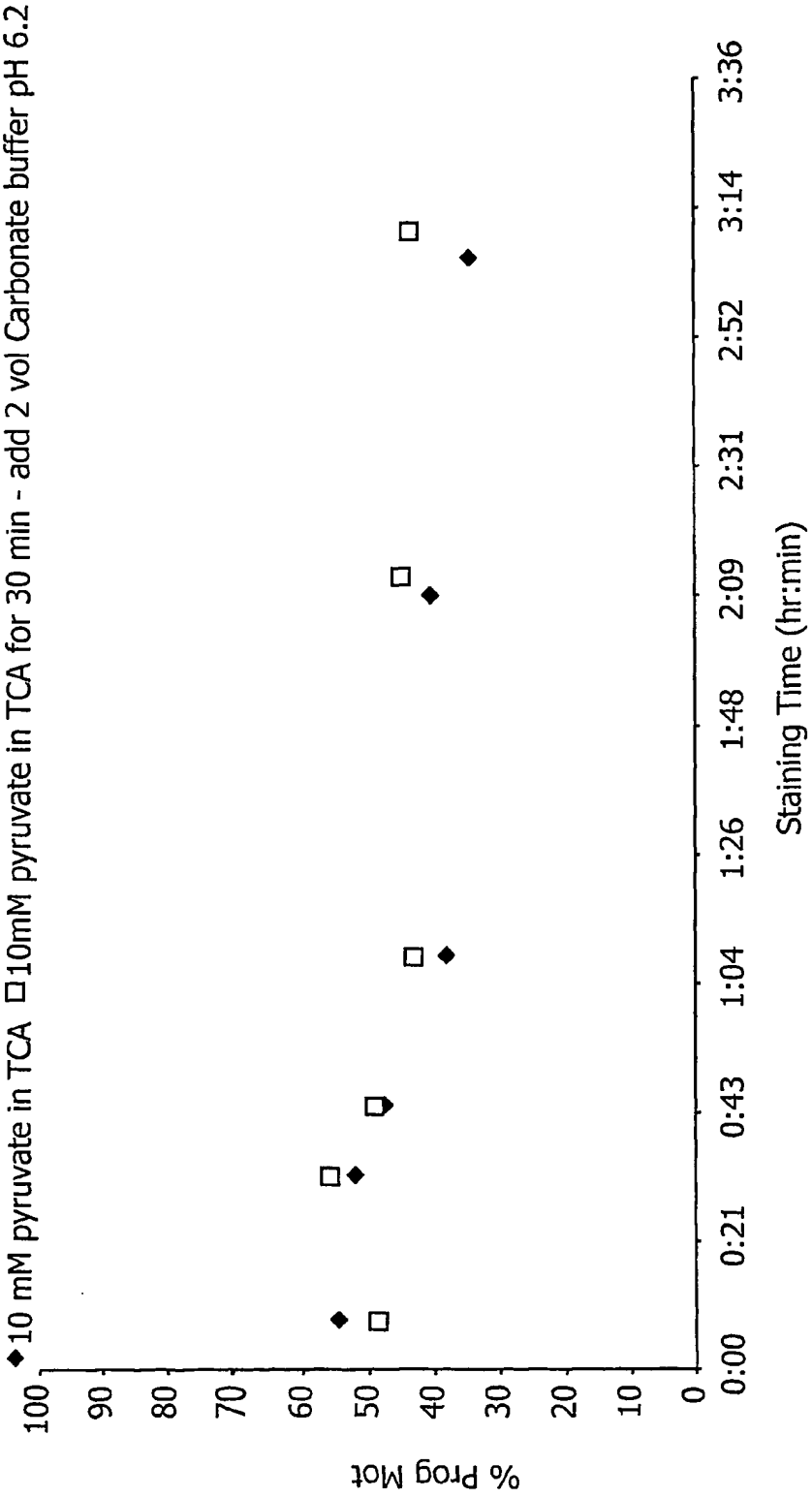

◆10 mM pyruvate in TCA □10mM pyruvate in TCA for 30 min - add 2 vol Carbonate buffer pH 6.2
% Prog Mot
100
90
80
70
60
50
40
30
20
10
0
0:00
0:21
0:43
1:04
1:26
1:48
2:09
2:31
2:52
3:14
3:36
Staining Time (hr:min)

FIG. 8

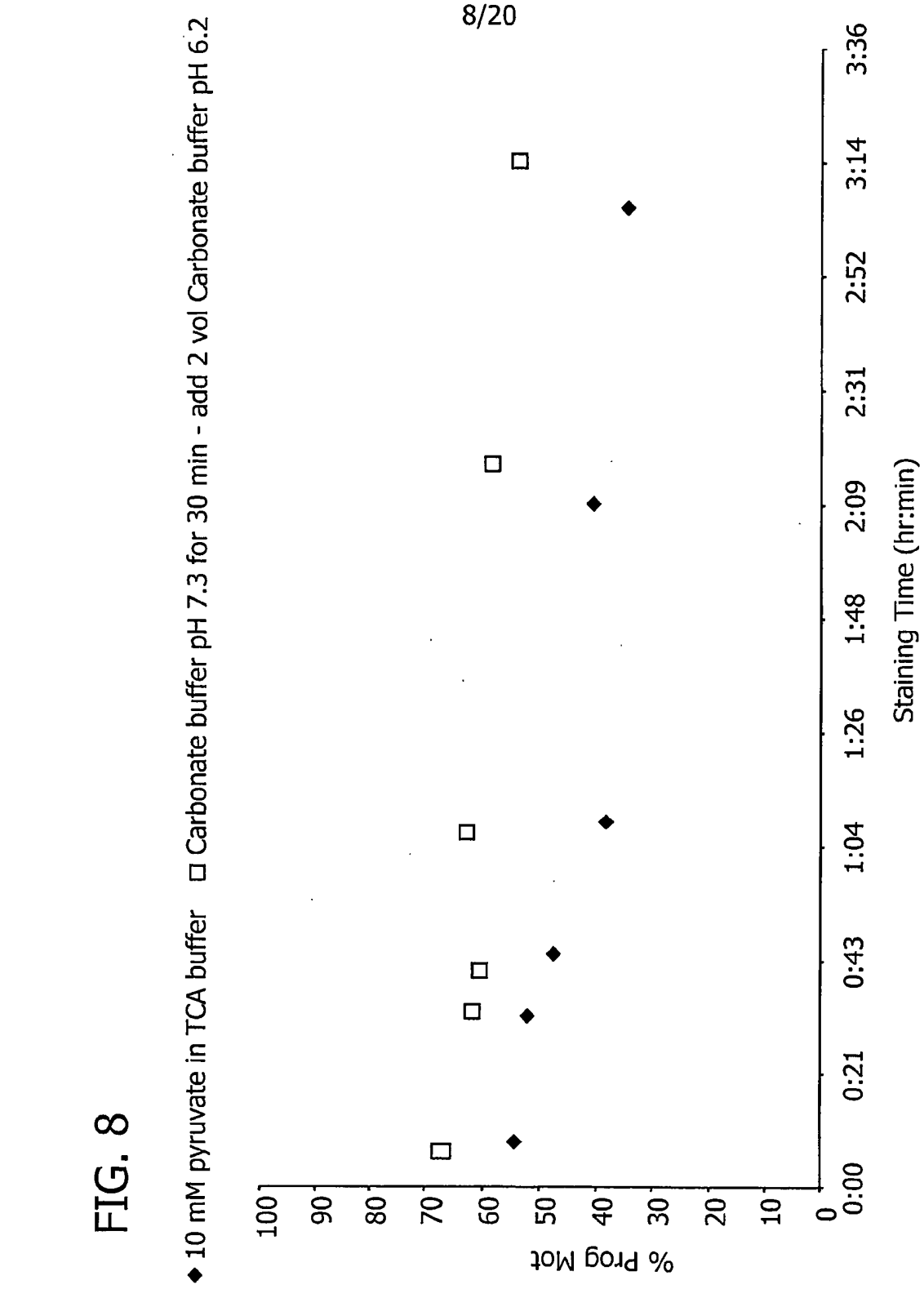

◆ 10 mM pyruvate in TCA buffer
□ Carbonate buffer pH 7.3 for 30 min - add 2 vol Carbonate buffer pH 6.2
% Prog Mot
100
90
80
70
60
50
40
30
20
10
0
0:00
0:21
0:43
1:04
1:26
1:48
2:09
2:31
2:52
3:14
3:36
Staining Time (hr:min)

FIG. 9

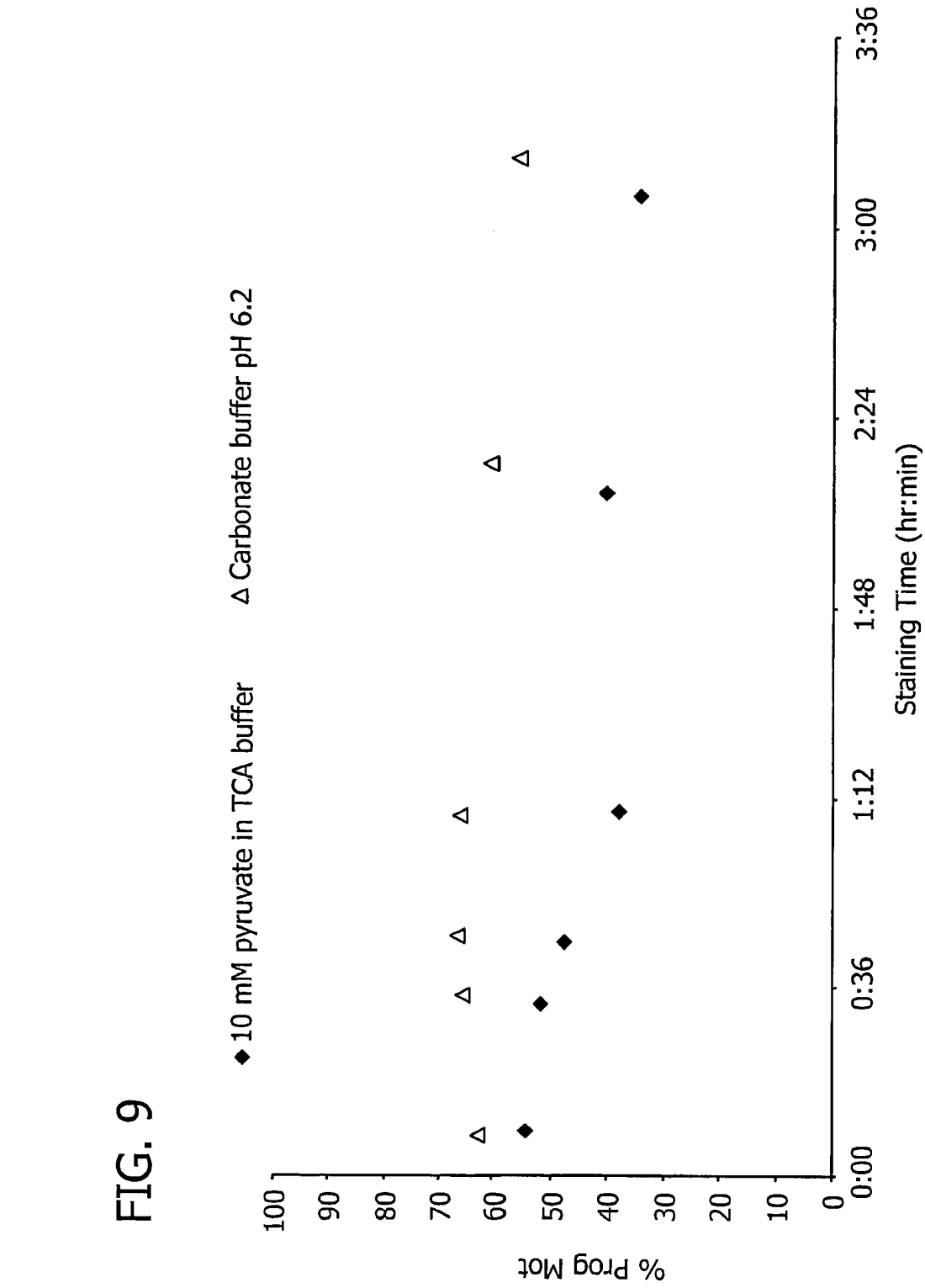

◆ 10 mM pyruvate in TCA buffer
Δ Carbonate buffer pH 6.2
% Prog Mot
100
90
80
70
60
50
40
30
20
10
0
0:00
0:36
1:12
1:48
2:24
3:00
3:36
Staining Time (hr:min)

FIG. 10

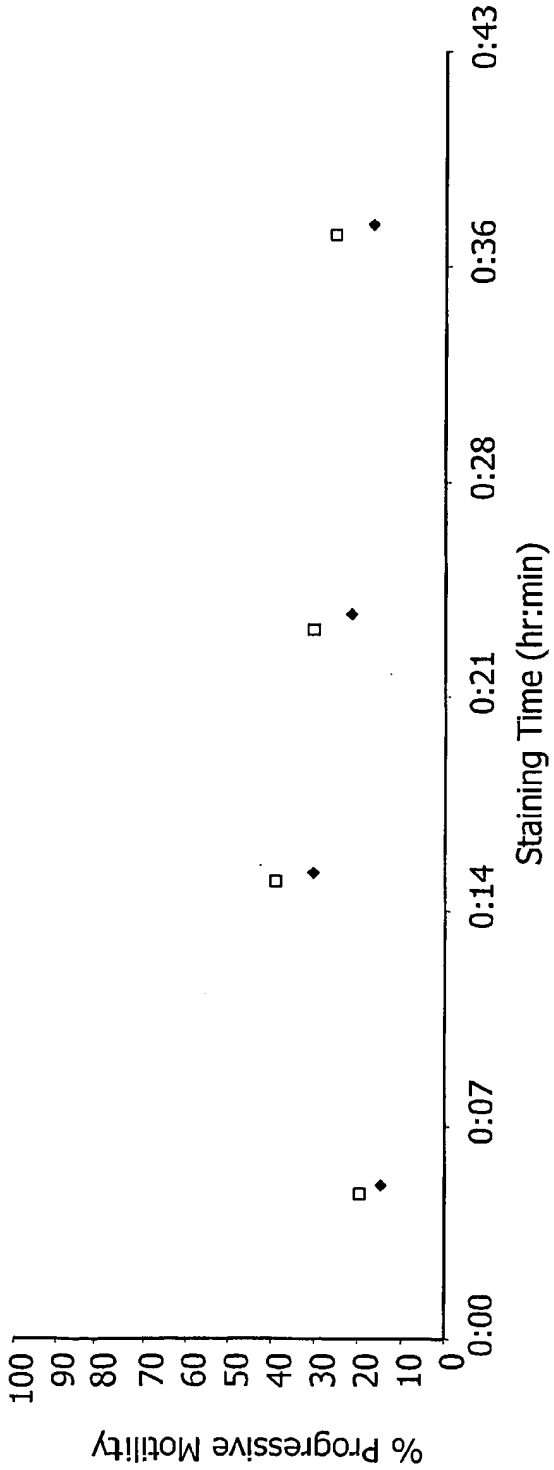

TCA Buffer
10 mM pyruvate in TCA buffer
% Progressive Motility
100
90
80
70
60
50
40
30
20
10
0
0:00
0:07
0:14
0:21
0:28
0:36
0:43
Staining Time (hr:min)

FIG. 11

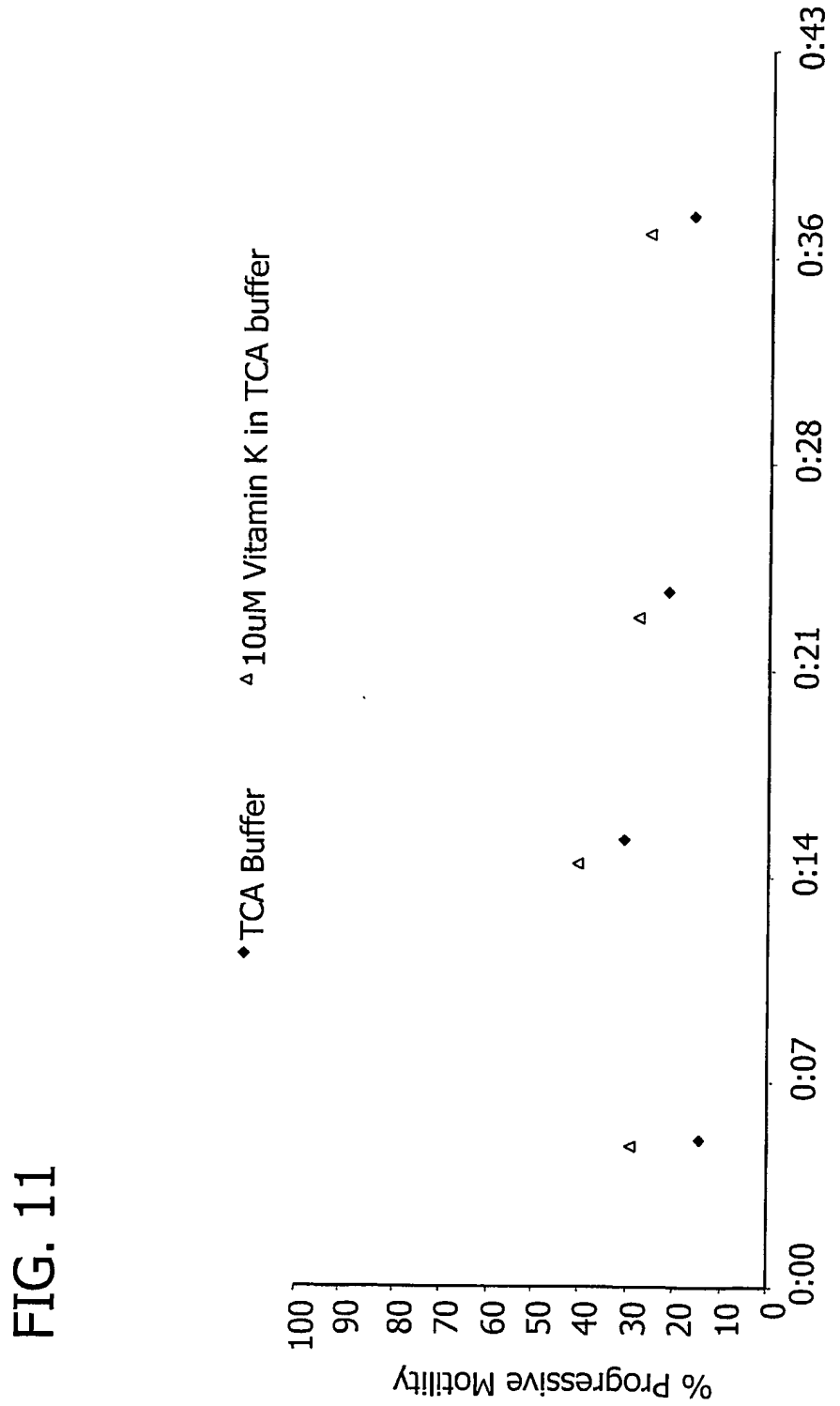

◆TCA Buffer
△10uM Vitamin K in TCA buffer
% Progressive Motility
100
90
80
70
60
50
40
30
20
10
0
0:00
0:07
0:14
0:21
0:28
0:36
0:43
Staining Time (hr:min)

FIG. 12

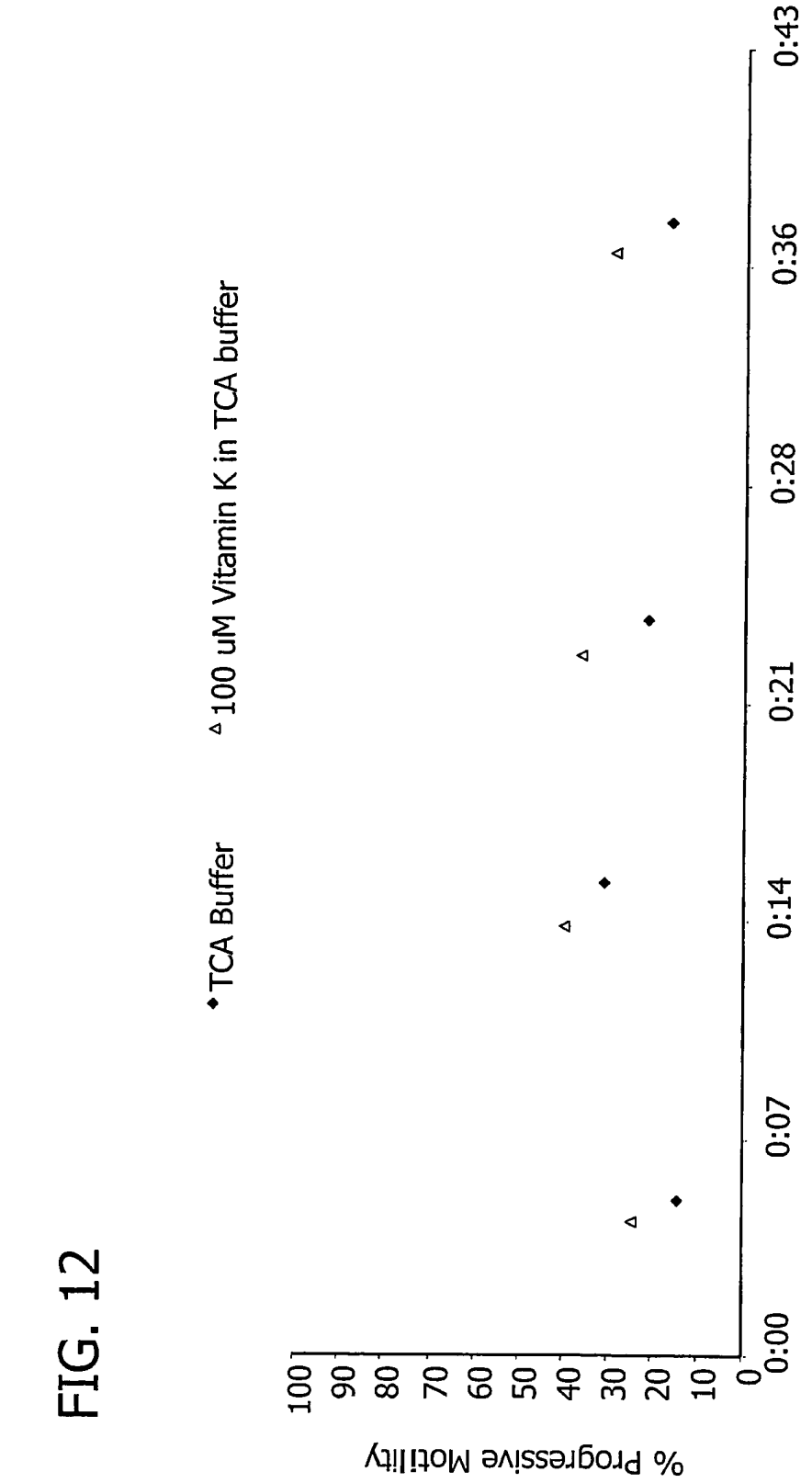

TCA Buffer
100 uM Vitamin K in TCA buffer
% Progressive Motility
100
90
80
70
60
50
40
30
20
10
0
0:00
0:07
0:14
0:21
0:28
0:36
0:43
Stain Time

FIG. 13

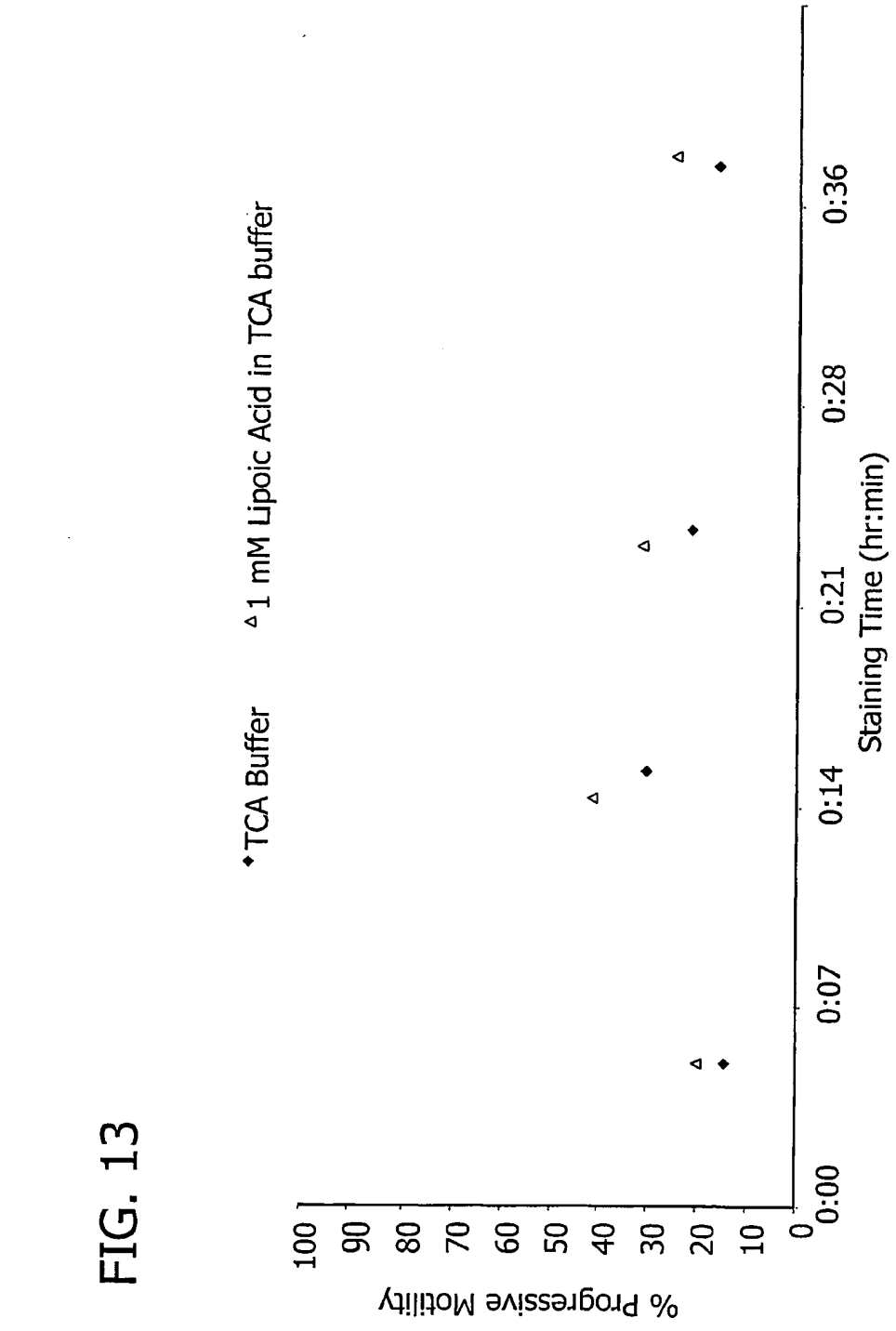

TCA Buffer
1 mM Lipoic Acid in TCA buffer
% Progressive Motility
100
90
80
70
60
50
40
30
20
10
0
0:00
0:07
0:14
0:21
0:28
0:36
Staining Time (hr:min)

FIG. 14

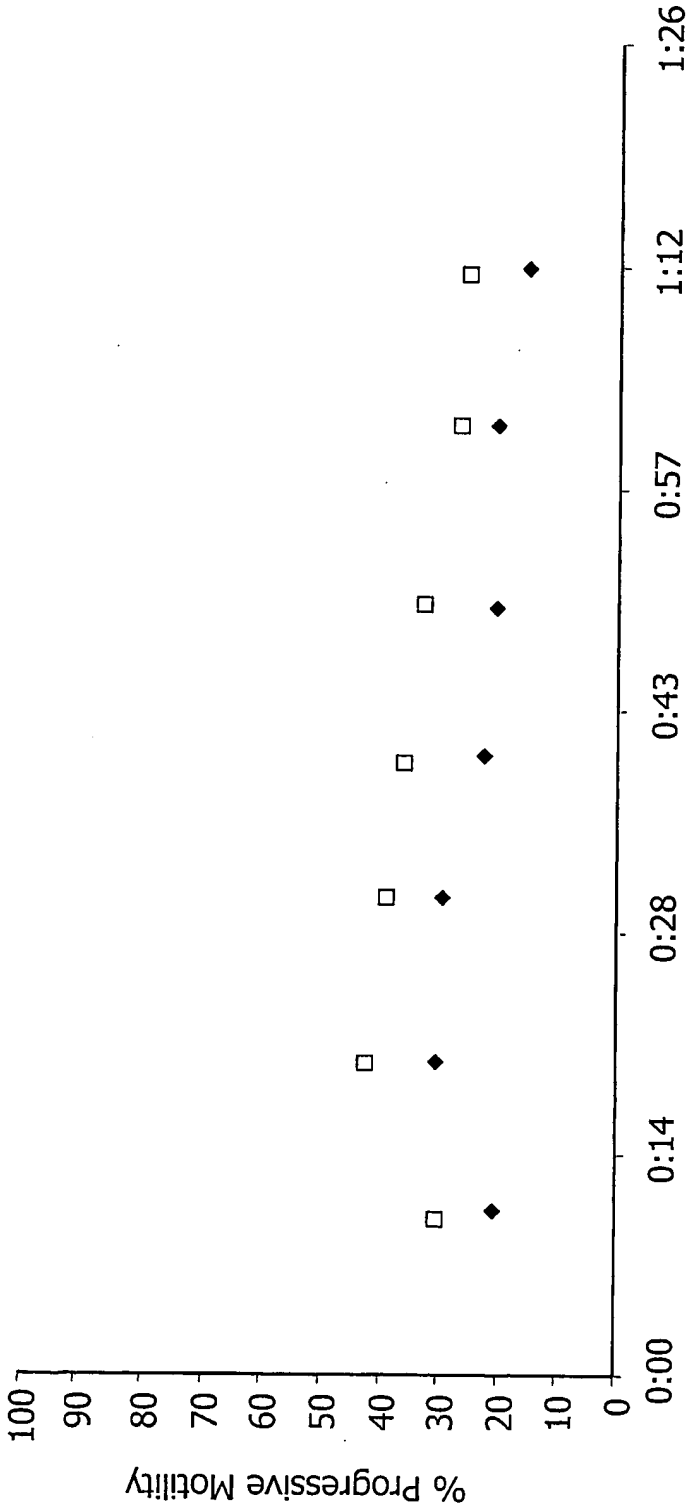

TCA buffer
10 mM pyruvate in TCA buffer
% Progressive Motility
100
90
80
70
60
50
40
30
20
10
0
0:00
0:14
0:28
0:43
0:57
1:12
1:26
Staining Time (hr:min)

FIG. 15

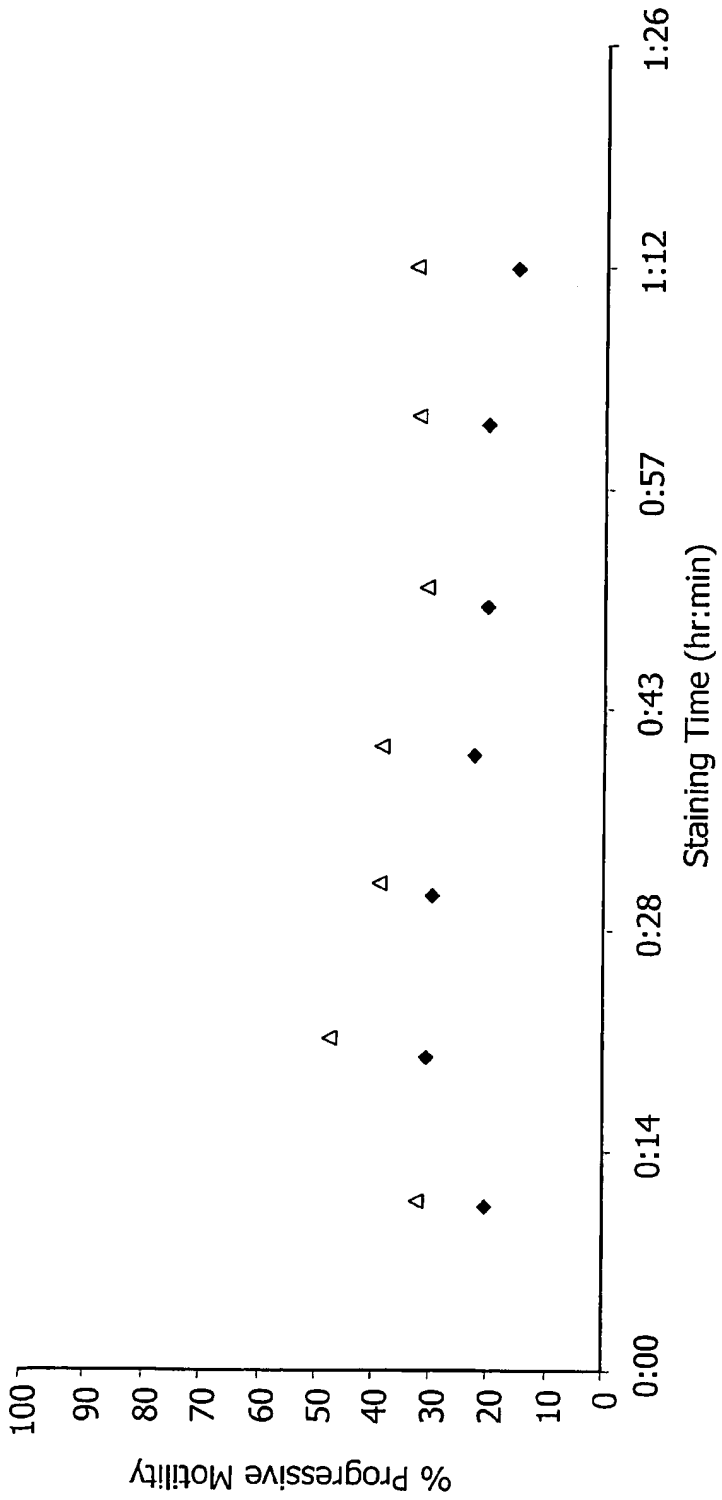

♦TCA buffer
△100 uM Vitamin K in TCA buffer
% Progressive Motility
100
90
80
70
60
50
40
30
20
10
0
0:00
0:14
0:28
0:43
0:57
1:12
1:26
Staining Time (hr:min)

FIG. 16

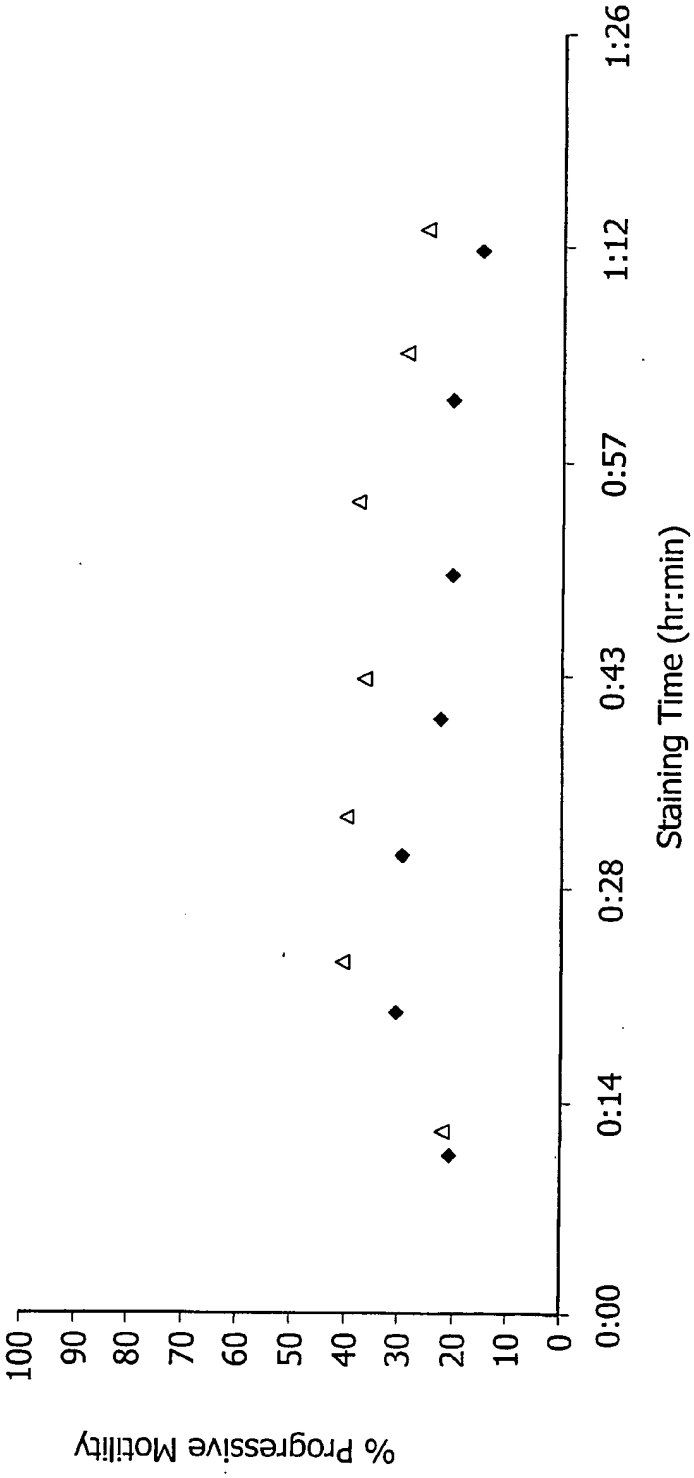

TCA buffer
1 mM Lipoic Acid in TCA buffer
% Progressive Motility
100
90
80
70
60
50
40
30
20
10
0
0:00
0:14
0:28
0:43
0:57
1:12
1:26
Staining Time (hr:min)

FIG. 17

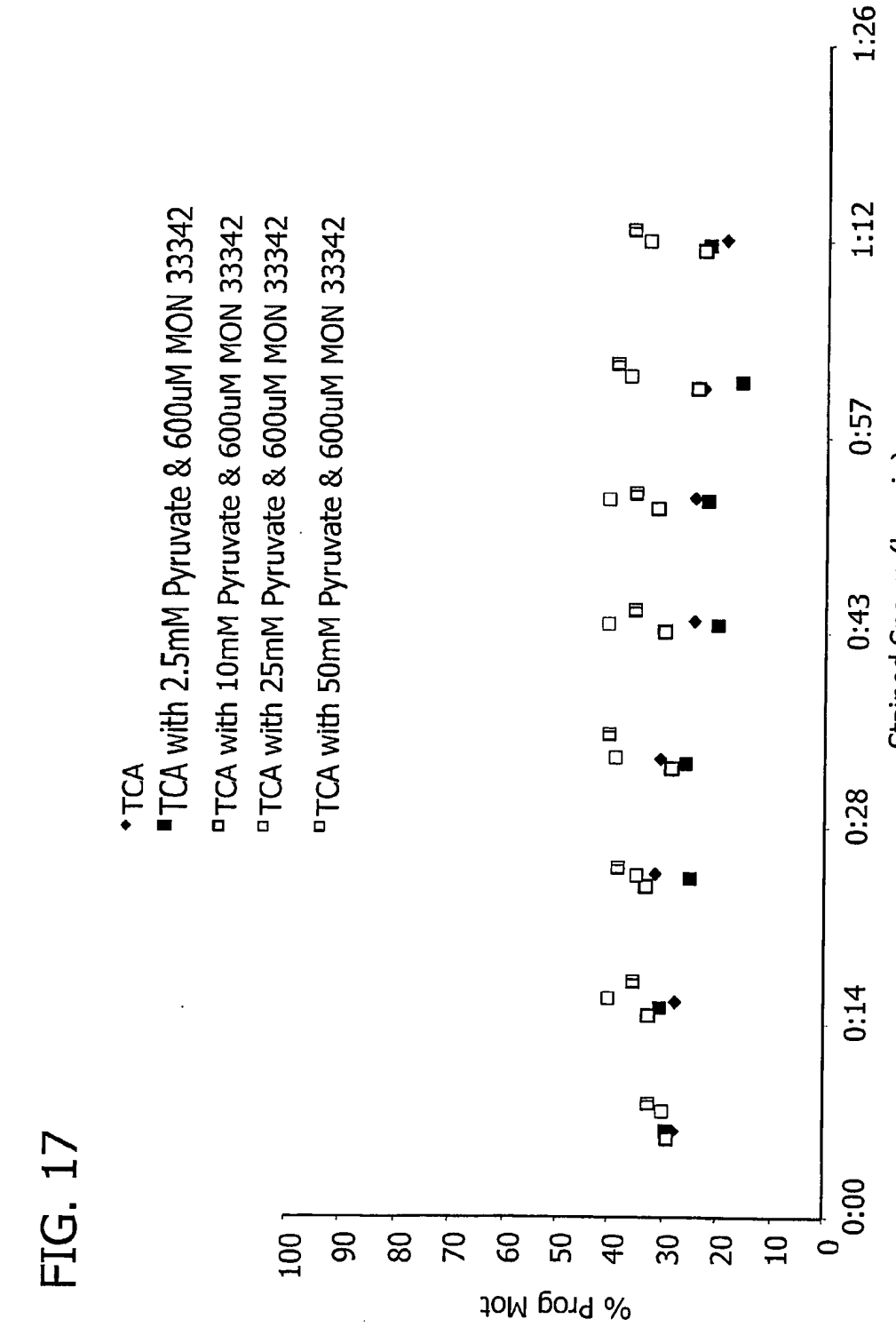

TCA
TCA with 2.5mM Pyruvate & 600uM MON 33342
TCA with 10mM Pyruvate & 600uM MON 33342
TCA with 25mM Pyruvate & 600uM MON 33342
TCA with 50mM Pyruvate & 600uM MON 33342
% Prog Mot
100
90
80
70
60
50
40
30
20
10
0
0:00
0:14
0:28
0:43
0:57
1:12
1:26
Stained Sperm (hr:min)

FIG. 18

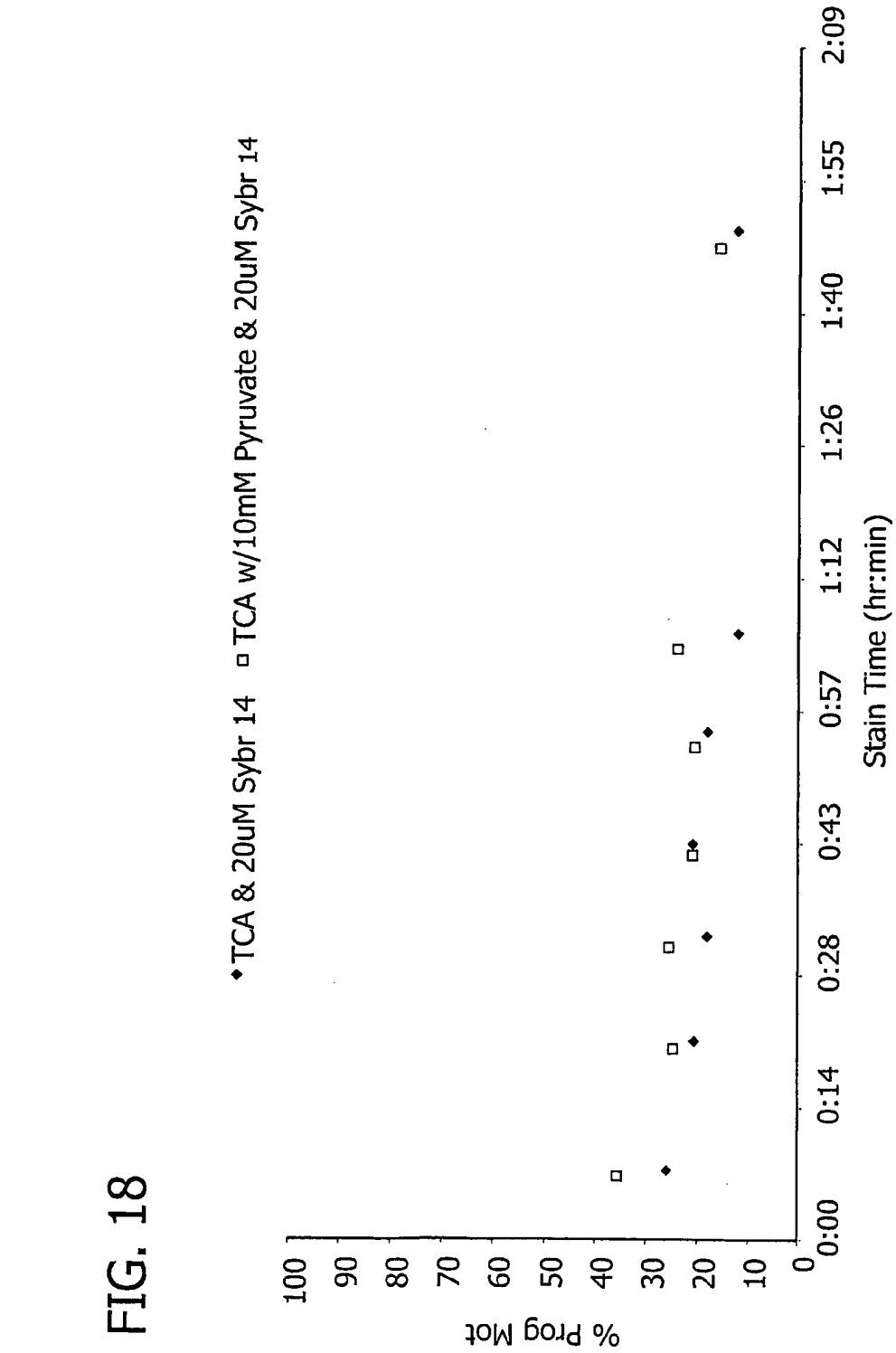

♦TCA & 20uM Sybr 14 □TCA w/10mM Pyruvate & 20uM Sybr 14
% Prog Mot
100
90
80
70
60
50
40
30
20
10
0
0:00
0:14
0:28
0:43
0:57
1:12
1:26
1:40
1:55
2:09
Stain Time (hr:min)

FIG. 19

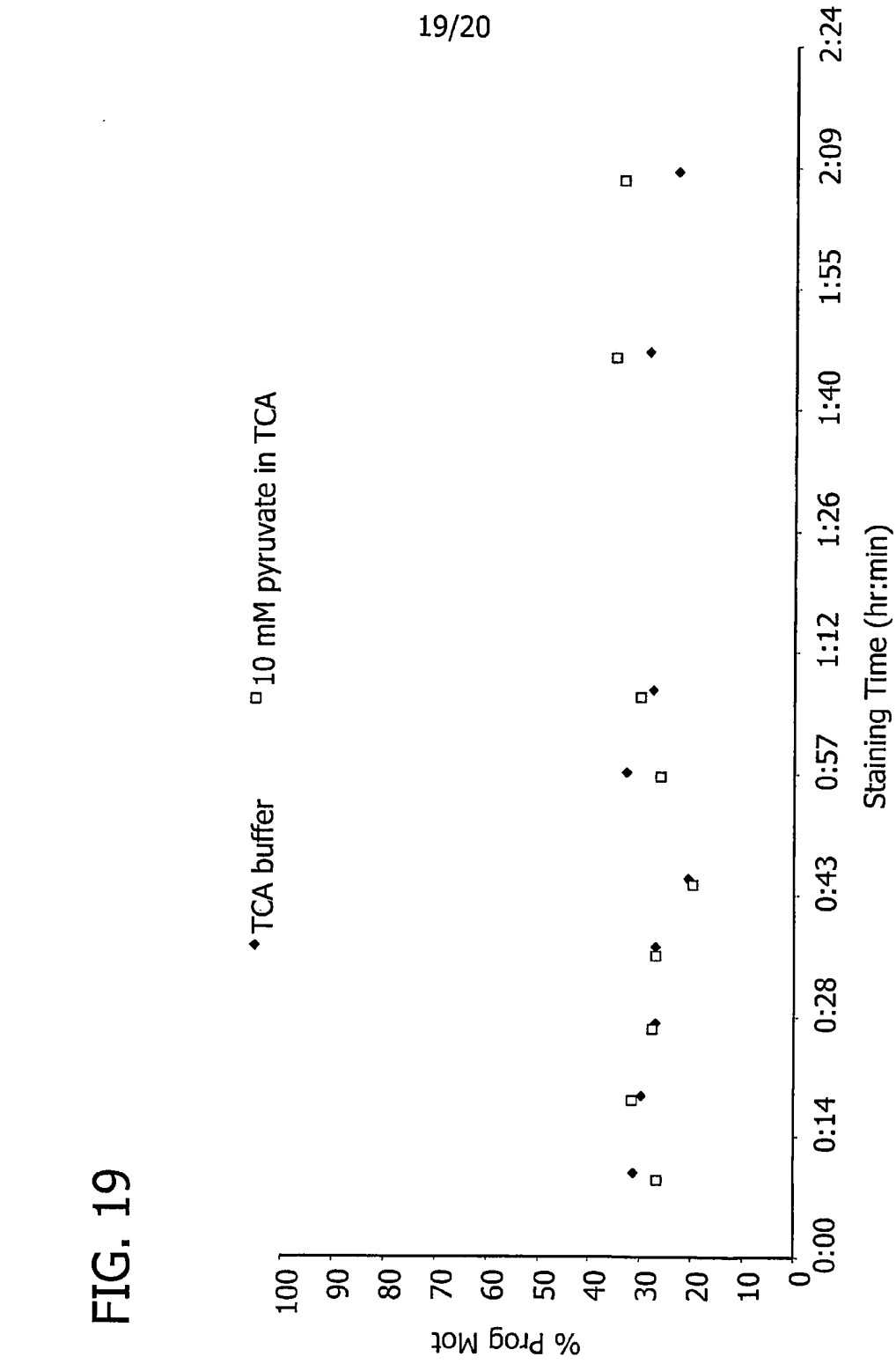
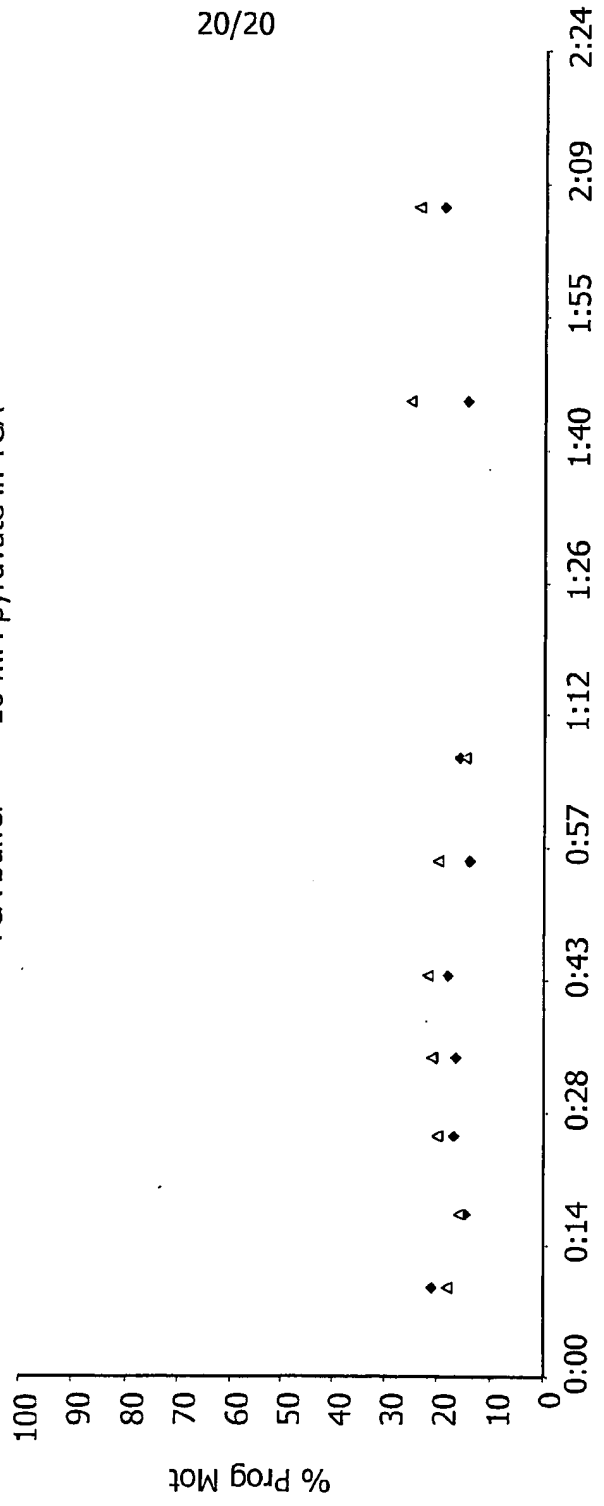

% Prog Mot
100
90
80
70
60
50
40
30
20
10
0
0:00
0:14
0:28
0:43
0:57
1:12
1:26
1:40
1:55
2:09
2:24
Staining Time (hr:min)
TCA buffer
10 mM pyruvate in TCA

FIG. 20


◆ TCA buffer
△ 10 mM pyruvate in TCA
% Prog Mot
100
90
80
70
60
50
40
30
20
10
0
0:00
0:14
0:28
0:43
0:57
1:12
1:26
1:40
1:55
2:09
2:24
Staining Time (hr:min)